Remarks

Claims 1-49 are pending.

The present invention is directed to methods of identifying variant recombinases that mediate recombination at variant recombination sites and producing site-specific recombination of DNA. The methods for identification of variant recombinases comprise, *inter alia*, a) bringing into contact a mutant recombinase, a first nucleic acid sequence comprising a first reporter gene and first and second recombination sites, *wherein the first and second recombination sites are variant recombination sites,* and a second nucleic acid sequence comprising a second reporter gene and third and fourth recombination sites, wherein the third and fourth recombination sites can be recombined by a non-mutant recombinase. It is important to note that the first and second sites are ***both*** variant recombinant sites.

Rejection Under 35 U.S.C. § 112, first paragraph

Claims 1-47 and 49 were rejected under 35 U.S.C. § 112, first paragraph, as not being enabled. Applicants respectfully traverse this rejection to the extent that it is applied to the claims as amended.

i. The Legal Standard

The Court of Appeals for the Federal Circuit (CAFC) has described the legal standard for enablement under § 112, first paragraph, as whether one skilled in the art could make and use the claimed invention from the disclosures in the patent coupled with information known in the art, without undue experimentation (*See, e.g., Genentech, Inc. v. Novo Nordisk A/S*,

108 F3d at 165, 42 USPQ2d at 1004 (quoting *In re Wright*, 999 F.2d 1557, 1561, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993); See also *In re Fisher*, 427 F.2d at 839, 166 USPQ at 24; *United States v. Telectronics, Inc.*, 857 F.2d 778 (Fed. Cir. 1988); *In re Stephens,* 529 F.2d 1343 (CCPA 1976)). The fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation (*M.I.T. v. A.B. Fortia*, 774 F.2d 1104 (Fed. Cir. 1985)). In addition, as affirmed by the Court in *Spectra-Physics, Inc. v. Coherent, Inc.*, 827 F.2d 1524 (Fed. Cir. 1987), a patent need not teach, and preferably omits, what is well known in the art.

Whether the disclosure is enabling is a legal conclusion based upon several underlying factual inquiries. See *In re Wands*, 858 F.2d 731, 735, 736-737, 8 USPQ2d 1400, 1402, 1404 (Fed. Cir. 1988). As set forth in *Wands*, the factors to be considered in determining whether a claimed invention is enabled throughout its scope without undue experimentation include the quantity of experimentation necessary, the amount of direction or guidance presented, the presence or absence of working examples, the nature of the invention, the state of the prior art, the relative skill of those in the art, the predictability or unpredictability of the art, and the breadth of the claims. In cases that involve unpredictable factors, "the scope of the enablement obviously varies inversely with the degree of unpredictability of the factors involved." *In re Fisher*, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970). The fact that some experimentation is necessary does not preclude enablement; what is required is that the amount of experimentation 'must not be unduly extensive.' *Atlas Powder Co., v. E.I. DuPont De Nemours*

& Co., 750 F.2d 1569, 1576, 224 USPQ 409, 413 (Fed. Cir. 1984). There is no requirement for examples.

ii. Rejection of claims 1-47 and 49 under 35 U.S.C. § 112, first paragraph

The Examiner has provided references to support his assertion that gene therapy is a new and developing art, lacks efficacy, and often results in adverse short term effects. However, the Applicants are **not** claiming methods directed to gene therapy. Instead, what is claimed are methods of identifying variant recombinases. Therapy is not the intended use (nor is the term used in any of the pending claims) for the methods as claimed. The prior art cited by the Examiner is drawn to the efficacy of gene therapy, a collection of methodologies for the efficient genetic modification of somatic cells to give a particular therapeutic outcome. The cited references point out that it is critical to modify a majority or all of the somatic cells of the organism. In contrast, for the *in vivo* practice of the present claims in an animal, modification of the germline by a standard transgenic approach guarantees that a given DNA be present in the cells of the animal. Using standard, well known methods, genes can be placed at genomic loci, resulting in reliable patterns of gene expression. For example, placement of a loxP reporter DNA at the ROSA26 locus in the mouse gives a reliable and reproducible expression of a reporter gene whose expression changes upon site-specific DNA recombination (see enclosed reference by Soriano *et al.*, 1999, *Nat. Genetics* 21:70). Moreover, because it is the phenotype of the cells in the organism that is important, and not necessarily the phenotype of the organism as a whole, efficient means to deliver genes into somatic cells would be sufficient to practice the

invention. This has been achieved, for example, by using genetically modified viruses to express Cre recombinase (see enclosed reference by Badorf *et al.,* 2002, *Genesis*, 33:119). The gene therapy problems, presented in the documents cited by the Examiner, speak to therapeutic benefit by changing the phenotype of the organism as a whole. This is not necessary to be able to detect a change in recombination status in individual cells of that organism. The somatic cell delivery problems of gene therapy (host immune problems, efficient DNA delivery to individual cells, etc.) are not applicable when using a transgenic animal approach that gives germline modification.

Several site-specific recombinases have already been shown to catalyze recombination in multicellular organisms after a first demonstration that they catalyze recombination in cultured cells. For example Cre in mice (see enclosed article by Lakso *et al., Proc. Natl. Acad. Sci.* USA 89:6232-6236; also see enclosed Sauer article, *Methods* 14:381; and see enclosed article by Metzger *et al.*, 1999, *Curr. Opin. Biotechnol.* 10:470); Cre in plants (see enclosed Odell *et al.,* U.S. Patent No. 5,658,772); Flp in mice (Dymecki, S., 1996, *Proc. Natl. Acad. Sci.* USA 93:6191; phiC31 Int in mice (see enclosed abstract by Olivares *et al.*, 2002, *Nat. Biotechnol.* 20:1124). Again, for the purposes of the presently pending claims, it is necessary to only monitor the state of individual cells (i.e. did recombination occur or not?). The phenotype of the organism as a whole is not a critical factor in monitoring the state of individual cells. As indicated by the enclosed reference by Soriano (*Nat. Genetics*, January 1999, Volume 21:70), the

ROSA26 "Cre reporter" mouse carries two loxP sites with a reporter gene and gives reliable and predictable reporting of Cre-mediated recombination in mice.

In view of the foregoing discussion, the pending claims are clearly enabled in view of what was commonly known to one of ordinary skill in the art at the time of filing the present application in combination with the present application.

Rejection Under 35 U.S.C. § 102

Claims 1-6 and 21 were rejected under 35 U.S.C. § 102(b) as being anticipated by *Cell*, Vol. 20, pp. 721-729 (1980) by Miller *et al.* ("Miller"). Applicants respectfully traverse this rejection.

i. The Legal Standard.

For a rejection of claims to be properly founded under 35 USC §102, it must be established that a prior art reference discloses each and every element of the claims. *Hybritech Inc v Monoclonal Antibodies Inc*, 231 USPQ 81 (Fed. Cir. 1986), *cert. denied*, 480 US 947 (1987); *Scripps Clinic & Research Found v Genentech Inc*, 18 USPQ2d 1001 (Fed. Cir. 1991). The Federal Circuit held in *Scripps*, 18 USPQ2d at 1010:

> Invalidity for anticipation requires that all of the elements and limitations of the claim are found within a single prior art reference. . . *There must be no difference* between the claimed invention and the reference disclosure, as viewed by a person of ordinary skill in the field of the invention. (Emphasis added)

A reference that fails to disclose even one limitation will not be found to anticipate, even if the missing limitation could be discoverable through further experimentation. As the Federal Circuit held in *Scripps, Id.*:

> [A] finding of anticipation requires that all aspects of the claimed invention were already described in a single reference: a finding that is not supportable if it is necessary to prove facts beyond those disclosed in the reference in order to meet the claim limitations. The role of extrinsic evidence is to educate the decision-maker to what the reference meant to persons of ordinary skill in the field of the invention, not to fill in the gaps in the reference.

For a prior art reference to anticipate a claim, it must enable a person skilled in the art to practice the invention. The Federal Circuit held that "a §102(b) reference must sufficiently describe the claimed invention to have placed the public in possession of it. . . [E]ven if the claimed invention is disclosed in a printed publication, that disclosure will not suffice as prior art if it was not enabling." *Paperless Accounting Inc v Bay Area Rapid Transit Sys.*, 231 USPQ 649, 653 (Fed. Cir. 1986) (citations omitted).

ii. Rejection of claims 1-6 and 21 under 35 U.S.C. § 102(b).

The Examiner points to page 725, column 2 (the Discussion), wherein lambda integrase activity is manifested under two conditions: in bacterial mutants that fail to support lambda site-specific recombination, and under conditions where the recombinational *att* sites are altered. However, one **MUST** look to the body of the paper (i.e. the data) in order to properly define "the

recombinational att sites" being discussed and to define precisely which sites are actually being recombined. The applicants respectfully submit that in **ALL** of the data presented by Miller, recombination proceeds between a wild type site and a variant site. For example, Miller teaches the isolation and characterization of an int protein that is active in a variety of mutants normally defective in lambda site-specific recombination. Lambda-promoted gal expression is dependent upon the lamba N protein. It should be noted that the secondary sites (i.e. those suggested by the Examiner as being "variant") are the same as those used by lambda-int$^+$. Therefore, by definition, the secondary sites of Miller are not variant recombination sites that ***are not*** recognized by non-mutant recombinase (see page 4, lines 5-8, of the present application, wherein "the constructs contain variant recombination sites that are not recognized by non-mutant recombinase but will undergo recombination in the presence of a mutant recombinase with altered specificity"). With regard to the non-paradigm att sites, the data supplied in column 2, of page 725, is clearly directed to recombination between one variant site and one non-variant site (i.e. "One variant carries the att24 mutation in attL and the other in attR.....17% for lambda-attL24-attR and to 11% for lambda-attL-attR24."). However, the claims as pending are directed to, in part, "a first nucleic acid sequence comprising a first reporter gene and first and second recombination sites, *wherein the first* ***and*** *second recombination sites* ***are variant recombination sites***" (emphasis added). In all of the cases presented in Miller, recombination proceeds between a wild type site and a variant site. Therefore, the cited passage at page 725, column 2 (the

Discussion), wherein lambda integrase activity is manifested under certain conditions, *does not* pertain to situations wherein **BOTH** sites are altered.

Claims 1-6 and 21 were rejected under 35 U.S.C. § 102(b) as being anticipated by *J. Mol. Biol.* (1990), 216, pp. 633-643, by Ackroyd *et al.* ("Ackroyd"). Applicants respectfully traverse these rejections.

The Examiner has asserted that the abstract of Ackroyd teaches a Tn3 resolvase is mutated by changing an amino acid to a corresponding amino acid from Tn21. The applicants respectfully submit that Ackroyd ***DOES NOT*** teach any Tn3 *mutant* resolvase. The abstract of Ackroyd is directed to mutating the Tn21 resolvase, not Tn3. The Tn3 resolvase that is tested throughout Ackroyd, is **WILD-TYPE**. *Ackroyd teaches the ability of* ***Tn21 resolvase mutants*** *to carry out site-specific recombination between res sites from either Tn21 or Tn3.* Nowhere does Ackroyd teach "mutated Tn3 resolvases retain their ability to recombine at the wild-type Tn3 and are now able to recombine at wild-type Tn21 recombination site" (see page 11 of the Office Action mailed on April 9, 2003).

Again, the transformants that harbor pEAK6 (wild type Tn21) *or any of its mutants* all yielded approximately the same number of colonies in the presence and absence of tetracycline (see Table 1 and page 638, second column, of Ackroyd). With the exception of the E173L mutant, each of the mutants gave virtually the same levels of recombination with regard to Tn21 res (pAA3) (i.e. the same or reduced activity as wild-type). Please note that the Tn3 resolvase is a wild-type resolvase, and that the data presented in Ackroyd clearly teaches variant resolvases

that recognize DNA not unlike their wild-type counterpart(s). Variant sites, as defined by the presently pending claims, are not recognized by non-mutant recombinases (see page 4, lines 5-8). The ability of the Tn21 resolvase and each of the mutants to provide 100% recombination of Tn21 res (E173L = 7%) is a direct contradiction of what the present application teaches. Ackroyd fails to identify ANY mutant recombinase that can recognize ANY **NEW** recombination site. Again, all sites used in Ackroyd are either wild type Tn21 res sites or wild type Tn3 res sites. Additionally, Ackroyd compares wild type and mutant recombinase activity, on two different sites, in a sequential manner *in different cells*.

Rejection Under 35 U.S.C. § 103

Claims 1-30, 32-45 and 47-49 were rejected under 35 U.S.C. § 103(a) as being unpatentable over by *Cell*, Vol. 20, pp. 721-729 (1980) by Miller *et al.* ("Miller"), or *J. Mol. Biol.* (1990), 216, pp. 633-643, by Ackroyd *et al.* ("Ackroyd"), in view of U.S. Patent No. 5,677,177 to Wahl et al. ("Wahl"). Applicants respectfully traverse these rejections.

i. The Legal Standard.

The U.S. Patent and Trademark Office has the burden under 35 U.S.C. § 103 to establish a *prima facie* case of obviousness. *In re Warner et al.*, 379 F.2d 1011, 154 U.S.P.Q. 173, 177 (C.C.P.A. 1967), *In re Fine,* 837 F.2d 1071, 1074, 5 U.S.P.Q.2d 1596, 1598-99 (Fed. Cir. 1988). In rejecting a claim under 35 U.S.C. § 103, the Examiner must establish a *prima facie* case that: (i) the prior art suggests the claimed invention; and (ii) the prior art indicates that the invention

would have a reasonable likelihood of success. *In re Dow Chemical Company,* 837 F.2d 469, 5 U.S.P.Q.2d 1529 (Fed. Cir. 1988).

The prior art must provide one of ordinary skill in the art with the motivation to make the proposed modifications needed to arrive at the claimed invention. *In re Geiger*, 815 F.2d 686, 2 U.S.P.Q.2d 1276 (Fed. Cir. 1987); *In re Lalu and Foulletier*, 747 F.2d 703, 705, 223 U.S.P.Q. 1257, 1258 (Fed. Cir. 1984). Claims for an invention are not *prima facie* obvious if the primary references do not suggest all elements of the claimed invention and the prior art does not suggest the modifications that would bring the primary references into conformity with the application claims. *In re Fritch*, 23 U.S.P.Q.2d, 1780 (Fed. Cir. 1992). *In re Laskowski*, 871 F.2d 115 (Fed. Cir. 1989). This is not possible when the claimed invention achieves more than what any or all of the prior art references allegedly suggest, expressly or by reasonable implication.

The Court of Appeals for the Federal Circuit recently warned that "the best defense against the subtle but powerful attraction of a hindsight-based obviousness analysis is rigorous application of the requirement for showing of the teaching or motivation to combine prior art references." In re Dembiczak, 175 F.3d 994 at 999 (Fed. Cir. 1999). While the suggestion to combine may be found in explicit or implicit teachings within the references, from the ordinary knowledge of those skilled in the art, or from the nature of the problem to be solved, the "question is whether there is something in the prior art as a whole to suggest the desirability, and thus the obviousness, of making the combination. WMS Gaming, Inc. v International Game Technology, 184 F.3d 1339 at 1355 (Fed. Cir. 1999). "The range of sources available, however,

does not diminish the requirement for actual evidence. That is, the showing must be clear and particular." In re Dembiczak, 175 F.3d 994 at 999 (Fed. Cir. 1999). Although with the answer in hand, the "solution" now appears obvious, that is not the test. The references must themselves lead those in the art to what is claimed. And in this case, there is simply no such teaching.

ii. Rejection of claims 1-30, 32-45 and 47-49 under 35 U.S.C. § 103(a)

Miller and Ackroyd

As stated in the foregoing discussion (under "Rejection Under 35 U.S.C. § 102), neither of Miller or Ackroyd teach the identification of a mutant recombinase, wherein the mutant recombinase recombines first and second sites that are both variant. Miller does not teach bringing a mutant Int recombinase together with a pair of mutant att sites. Instead, Miller teaches bringing together a mutant Int recombinase with a pair of att sites, one of which is mutant and the other is wild type. Ackroyd teaches the sequential testing of a mutant recombinase with each of two different pairs of recombination sites (i.e. first, test one pair of sites, and then test a second pair of sites; when done in cells, Ackroyd used separate cells (different bacterial strains)). This did not result in the identification of a recombinase that is able to recombine a variant recombination site.

Wahl

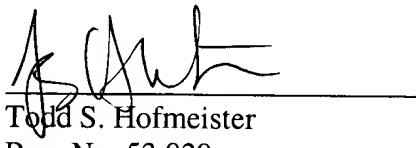
Wahl fails to teach a variant recombinase, or any alteration of substrate specificity. Therefore, even in combination, there is nothing that would lead one skilled in the art to the claimed methods.

Summary

There is no teaching in the prior art, individually or in combination, of a variant recombinase with altered specificity (i.e. recognizing sequences that are ***not*** recognized by non-mutant recombinases). Furthermore, there is no teaching of assessing the recombination of two sites, wherein the two sites are variant. The applicants enclosed a copy of a reference by Yoziyanov *et al.* (Nucleic Acids Res., 2002, Vol. 30, No.7, pages 1656-1663) with the response and amendment mailed on December 27, 2002. This reference, published after the filing date of this application, extols the novelty of using a double recombination reporter strategy, as claimed, and provides independent third party evidence of the novelty and non-obviousness of the claimed method.

Allowance of claims 1-43, 45 and 47-49 is respectfully solicited.

Respectfully submitted,



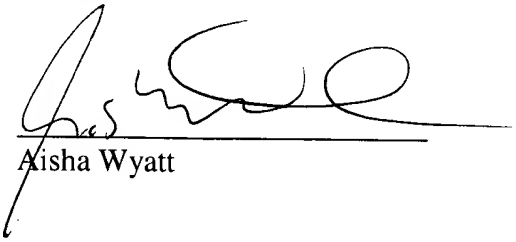
Todd S. Hofmeister
Reg. No. 53,029

Date: July 9, 2003

HOLLAND & KNIGHT LLP
One Atlantic Center, Suite 2000
1201 West Peachtree Street
Atlanta, Georgia 30309-3400
(404) 817-8473
(404) 817-8588 (Fax)

Certificate of Mailing Under 37 C.F.R. § 1.8(a)

I hereby certify that this paper, along with any paper referred to as being attached or enclosed, is being deposited with the United States Postal Service on the date shown below with sufficient postage as first-class mail in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.



Aisha Wyatt

Date: July 9, 2003

ATL1 #577191 v1

Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 6232–6236, July 1992
Genetics

Targeted oncogene activation by site-specific recombination in transgenic mice

(*cre/lox*/lens development/simian virus 40 large tumor antigen)

M. LAKSO*†, B. SAUER‡, B. MOSINGER, JR.*, E. J. LEE*, R. W. MANNING‡, S.-H. YU*, K. L. MULDER*, AND H. WESTPHAL*

*Laboratory of Mammalian Genes and Development, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892; and ‡Du Pont–Merck Pharmaceuticals, Inc., Experimental Station E328, Wilmington, DE 19880-0328

Communicated by Igor B. Dawid, April 9, 1992

ABSTRACT **An efficient and accurate method for controlled *in vivo* transgene modulation by site-directed recombination is described. Seven transgenic mouse founder lines were produced carrying the murine lens-specific αA-crystallin promoter and the simian virus 40 large tumor-antigen gene sequence, separated by a 1.3-kilobase-pair Stop sequence that contains elements preventing expression of the large tumor-antigen gene and Cre recombinase recognition sites. Progeny from two of these lines were mated with transgenic mice expressing the Cre recombinase under control of either the murine αA-crystallin promoter or the human cytomegalovirus promoter. All double-transgenic offspring developed lens tumors. Subsequent analysis confirmed that tumor formation resulted from large tumor-antigen activation via site-specific, Cre-mediated deletion of Stop sequences.**

A desired goal of transgene technology is efficient and accurate manipulation of DNA sequences after their integration in the germ line. DNA recombinases that mediate integration or excision of sequences at specific recognition sites in both prokaryotic (1–5) and eukaryotic (6–10) systems are well suited for this purpose. The bacteriophage P1 recombinase Cre catalyzes reciprocal recombination at a specific locus of crossing over (*lox*) (11–16). The *lox* sequence is composed of two 13-base-pair (bp) inverted repeats separated by an 8-bp spacer region. Upon binding to the inverted repeats, Cre synapses with a second *lox* site and then cleaves the DNA in the spacer region to initiate strand exchange with the synapsed *lox* partner. No additional factors are required in the recombination.

In this study, we examine the potential of the *cre/lox* system to activate a dormant transgene in the mouse. The simian virus 40 (SV40) large tumor antigens (TAgs) directed to the lens by a murine αA-crystallin promoter (mαA) cause malignant lens tumors (17). We inserted between mαA and TAg a specially designed Stop sequence that prevents gene expression and is flanked by *lox* sequences. By crossing the dormant TAg transgenic mouse lines with Cre-expressing transgenic lines, we report here that the Cre protein recognizes the *lox* sites of the mαA–Stop–TAg transgene and recombines the two *lox* sequences, thereby removing Stop and activating TAg. Our studies show that targeted transgene modification in the mouse can be performed efficiently and accurately with a prokaryotic recombinase.

MATERIALS AND METHODS

Recombinant DNA Constructs and Transgenic Mice. The mαA–Stop–TAg transgene was constructed from the previously described plasmid pαA366a-T (17) by inserting to the *Bam*HI site between mαA and TAg a 1.3-kbp Stop fragment flanked by directly repeated *lox* sequences (5′-ATAACTTCGTATAGCATACATTATACGAAGTTAT-3′) (Fig. 1). The Stop sequence was composed of the 550-bp C-terminal sequence of yeast *His3* gene, 825 bp of the SV40 polyadenylylation signal region, and a synthetic oligonucleotide (5′-GATCTGACAATGGTAAGTAAGCTT-3′, where **ATG** is a false translation initiation signal and GTAAGT is a 5′ splice donor site). The human cytomegalovirus (hCMV)–*cre* construct was obtained by fusing the 1.2-kbp *cre* gene to the hCMV promoter as described (16) and the mαA–*cre* construct was derived from the hCMV–*cre* construct by changing the promoter sequence and by a T to G substitution at the −3 position. Transgenic mice were produced as described (18).

Screening of Transgenic Mice. The genotypes of all offspring were analyzed both by PCR and by Southern blots. For PCR analysis, mouse tail DNAs (2 μg) were amplified by 35 cycles (1 min 20 sec, 92°C; 1 min, 65°C; 1 min 30 sec, 72°C) on a thermal cycler. The 5′ primer for *cre* was 5′-GGACATGTTCAGGGATCGCCAGGCG-3′ and the 3′ primer was 5′-GCATAACCAGTGAAACAGCATTGCTG-3′. The 5′ and 3′ primers for TAg were 5′-GGTCTTGAAAGGAGTGCCCTGGGGGA-3′ and 5′-CCTCAGTTGCATCCCAGAAGCCTCC-3′, respectively. Twenty percent of the reaction volume was analyzed on a 1.75% agarose gel. For Southern blot analysis, tail genomic DNAs (5 μg) were digested with *Bam*HI and separated on a 0.75% agarose gel. Transfer to GeneScreen filters (DuPont) and Southern hybridization were performed according to Maniatis *et al.* (19). A 2.2-kbp *Taq* I/*Bam*HI fragment of SV40 viral DNA (Bethesda Research Laboratories) and a 0.4-kbp *Bam*HI fragment of the 5′ portion of *cre* were used as hybridization probes. The expression levels of the *cre* transgenes were determined either by a functional test in fibroblasts and kidney cells derived from hCMV–*cre* transgenic mice (20) or by a PCR amplification of cDNAs synthesized from total RNAs from mαA–*cre* lenses. Lens RNAs (2 μg) were converted to single-strand cDNA by Moloney murine leukemia virus reverse transcriptase and were amplified by 35 cycles of PCR as described above.

Phenotype Analysis of Mouse Lenses. Mouse eyes were fixed in 4% paraformaldehyde or 4% glutaraldehyde and 36.8% formaldehyde, embedded in methacrylate, sectioned, and stained with hematoxylin and eosin as described (21). Immunoperoxidase staining with an antibody specific to TAg was performed on frozen tissue sections (17) under the conditions previously reported (22), except that nonspecific

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: SV40, simian virus 40; TAg, large tumor antigen; mαA, murine αA-crystallin promoter; hCMV, human cytomegalovirus promoter/enhancer.

†To whom reprint requests should be addressed.

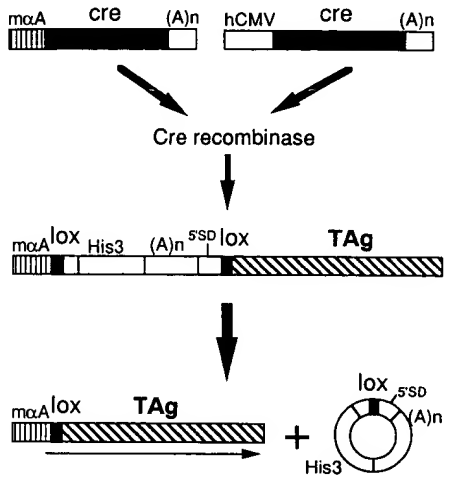


FIG. 1. DNA constructs used for generating transgenic mice. The *Nci* I fragment microinjected into fertilized mouse eggs is shown. mαA–Stop–TAg contains the mαA promoter separated from the SV40 TAg sequences by the 1.3-kbp regulatory Stop fragment, which was flanked by directly repeated *lox* sites and inserted into the *Bam*HI site between the mαA promoter and the TAg gene of the previously described plasmid pαA366a-T (17). The 3.3-kbp *Hin*dIII fragment of pBS187 plasmid (16) contained the *cre* gene under the control of the hCMV promoter/enhancer (hCMV–*cre*). The 3.0-kbp *Sma* I/*Hin*dIII fragment containing mαA promoter and *cre* gene (mαA–*cre*) was derived from pBS187 by changing the promoter sequence and by T to G substitution at the −3 position for translation enhancement (16). (A)n, polyadenylylation signal; 5'SD, 5' splice donor site.

binding was blocked with normal goat serum from the Vectastain detection kit (Vector Laboratories).

Molecular Analysis of Double-Transgenic Mouse Genotypes. Total genomic DNAs (1 μg) of eyes and/or tails from each mouse were amplified with a 5' primer (5'-GCTCCTGTCTGACTCACTGCCAG-3') identical to the mαA promoter sequence and a 3' primer (5'-CCCCCAGGCACTCCTTTCAAGACC-3') complementary to the TAg first exon sequence. Amplification occurred in 100 μl of PCR buffer with 1.6 mg of bovine serum albumin per ml by 35 cycles of PCR (1 min 20 sec, 96°C; 3 min 30 sec, 75°C with 5-sec automatic cycle extension). One-third volume of the PCR amplification products was loaded on 1.75% agarose gel for analysis. The 220-bp PCR amplification products were then isolated from the agarose gel by electroelution and cloned into pGEM4Z plasmid (Promega). Double-stranded DNAs were sequenced as described (23).

RESULTS AND DISCUSSION

The *cre/lox* Binary System. The design of our experiment calls for accumulation of chromosomal *lox* sites and active Cre recombinase in specific target cells of transgenic mice. In this constellation, the recombinase would excise a Stop signal that prevents expression of SV40 tumor antigens and would thereby initiate oncogenesis in the target tissue. The removal of even a single Stop sequence at an integration site that may contain more than one transgene copy would activate the oncogene.

Production of Transgenic Mice Carrying Either the Dormant TAg Oncogene or the Cre Recombinase Gene. The mαA–Stop–TAg dormant oncogene construct was designed to contain the 1.3-kbp Stop fragment inserted between mαA and TAg (Fig. 1). The Stop sequence contained a 550-bp spacer derived from C-terminal sequences of the yeast *HIS3* gene to enhance the efficiency of the downstream 825-bp SV40 polyadenylylation signal in terminating transcripts initiated by the mαA promoter. An additional safeguard to prevent TAg expression was a synthetic oligonucleotide, including a false translation initiation signal (ATG), and a 5' splice donor site (GTAAGT). The Stop fragment was flanked by directly repeated *lox* sequences. Cre expression constructs were obtained by fusing the 1.2-kbp *cre* gene to the mαA promoter (mαA–*cre*) or to the hCMV promoter (hCMV–*cre*). In the mαA–*cre* construct, a T to G nucleotide substitution was introduced to the −3 position relative to the AUG codon to enhance translation (16).

Transgenic mice were produced by injecting separately all three gene constructs as linear fragments into the pronuclei of fertilized mouse eggs that were then transferred to oviducts of pseudopregnant foster mothers (18). Seven independent transgenic mouse founder lines harboring the mαA–Stop–TAg construct exhibited normal translucent lenses, indicating that the Stop sequences effectively prevented TAg expression. Seven founder animals with the mαA–*cre* transgene and three founders with the hCMV–*cre* transgene were also obtained. No abnormalities among founders or their offspring were observed. High *cre* expressor lines were selected based (*i*) on the presence of functional Cre enzyme in fibroblasts and kidney cells from hCMV–*cre* transgenic lines or (*ii*) on a PCR analysis of cDNAs synthesized from total RNAs of mαA–*cre* lenses as described in *Materials and Methods*. F_1 progeny of two mαA–Stop–TAg founder lines, mαA–Stop–TAg1 with >50 copies and mαA–Stop–TAg2 with ≈50 copies of the transgene per haploid genome, were mated with those mαA–*cre* and hCMV–*cre* transgenic lines that showed high *cre* expression (Table 1).

Table 1. Transgenic mouse lines and occurrence of tumors

Genotype	No. of animals	No. of animals with lens tumors
Experiment 1*		
mαA–Stop–TAg1	70	0
mαA–Stop–TAg2	21	0
mαA–*cre*	>100	0
hCMV–*cre*	>100	0
mαA–Stop–TAg1/mαA–*cre*	17	17
mαA–Stop–TAg1/hCMV–*cre*	10	10
mαA–Stop–TAg2/mαA–*cre*	4	4
mαA–Stop–TAg2/hCMV–*cre*	5	5
Experiment 2†		
mαA–Stop–TAg1/mαA–*cre*	10	10
mαA–Stop–TAg1	9	0
mαA–*cre*	11	0
WT	10	0
Experiment 3‡		
mαA–Stop–TAg1/mαA–*cre*	16	16
mαA–Stop–TAg1	7	0
mαA–*cre*	8	0
WT	1	0

*The genotypes of the animals were determined by PCR and Southern blot analysis. From the seven transgenic mouse founder lines carrying the dormant mαA–Stop–TAg gene, two were selected for mating. The A9874 founder line of the mαA–*cre* lines was selected for its high *cre* expression based on lens RNA analysis by PCR. The hCMV–*cre* founder line DP769 was also chosen because of its high *cre* expression based on Cre activity as described. All 36 double-transgenic animals had cataracts and all single transgenics had normal eyes.

†Analysis of four successive litters of F_1 generation single transgenic mαA–*cre* female and F_1 generation single-transgenic mαA–Stop–TAg1 male. Genotypes of all offspring (n = 40) were confirmed by PCR and Southern analysis. WT, wild type.

‡Analysis of offspring from a cross between mαA–Stop–TAg1/mαA–*cre* double-transgenic female and male (produced by matings as described in †), which were heterozygous for each transgene. Genotypes of all offspring (n = 32) were analyzed by PCR. WT, wild type.

All Double-Transgenic Offspring Exhibit Lens Cataracts. The double-transgenic offspring harboring both the mαA–Stop–TAg and mαA–*cre* or hCMV–*cre* transgenes were easily identified on the basis of lens cataracts upon eye opening 10 days after birth (Fig. 2*B*). The genotypes of all offspring were confirmed by PCR (Fig. 3*A*) and Southern blot (Fig. 3*C*) analysis of DNA obtained from tail biopsies at weaning age. Two separate matings between mαA–Stop–TAg1 and mαA–*cre* progeny generated 17 double transgenics, each with cataracts in both lenses, from a total of 65 pups. Likewise, double transgenics resulting from crosses between mαA–Stop–TAg2 and mαA–*cre* or between mαA–Stop–TAg1 or -2 and hCMV–*cre* all had cataractous lenses at expected Mendelian frequencies (Table 1). More recently, crossing both *cre* transgenic lines with a third mαA–Stop–TAg transgenic founder line with fewer copies than the first two founder lines has also resulted in offspring that exhibited cataractous eyes at expected Mendelian frequencies (data not shown).

Malignant Transformation Results from TAg Expression. Histological analysis of lens sections obtained from double-transgenic animals consistently revealed morphological changes characteristic of proliferating lens tumors (Fig. 2*C*). Instead of the single mitotically active layer of anterior epithelial cells seen in normal lenses of mαA–Stop–TAg transgenic mice (data not shown), a multilayered heterogenous epithelium consisting of anaplastic cells was observed in the anterior part of the double-transgenic lenses. Tongues of invasive growth were extending into the rest of the disorganized lens mass. The lens mass was punctuated with large cysts that most likely result from improper elongation of fiber cells. Immunostaining of frozen lens sections with a polyclonal antibody to TAg showed a strong signal in the anaplastic cell mass (Fig. 2*D*). TAg could not be detected in lenses of mαA–Stop–TAg littermates (data not shown). Taken together, these results strongly suggest that the malignant transformation was due to TAg expression in the lenses of double-transgenic animals.

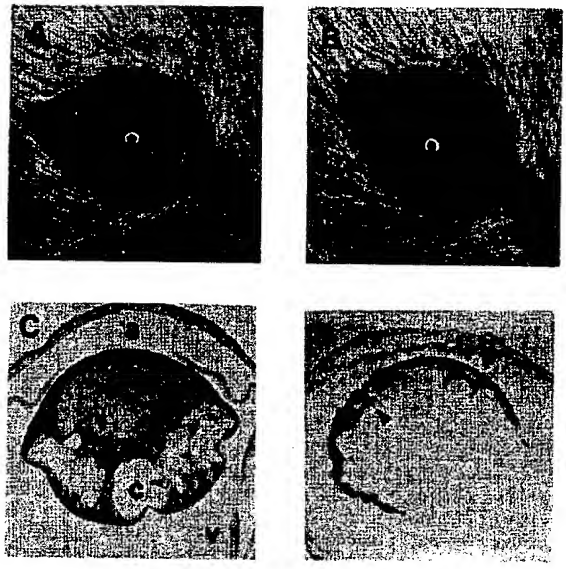


FIG. 2. Phenotype of double-transgenic mice bearing both mαA–Stop–TAg and mαA–*cre* genes. (*A*) Eye of a normal mαA–Stop–TAg1 single-transgenic mouse at 10 weeks of age. The albino mouse eye appears red. (*B*) Eye of a mαA–Stop–TAg1/mαA–*cre* double-transgenic sibling. (*C*) Lens pathology of a double-transgenic mouse. Hematoxylin and eosin-stained eye section from a 16-day-old mαA–Stop–TAg1/mαA–*cre* mouse. Aqueous humor (a), vitreous humor (v), cyst (c), and anaplastic cells (arrowhead) are shown. (×8.) (*D*) Indirect immunostaining with an antibody specific to TAg (22) of a lens section from the same animal as in *C*. Anterior epithelial cells (arrowhead) are strongly stained. The antibody did not stain lenses from single-transgenic littermates. (×6.)

TAg Activation Is Due to Site-Specific Recombination in the Embryonic Mouse Genome. The activation of the dormant TAg by Cre was assessed by PCR analysis of transgene sequences in affected and control lenses. A 24-bp 3′ primer complementary to the first exon sequence of TAg and a 23-bp 5′ primer identical to the mαA promoter sequence were synthesized. Amplifications with these primers predictably generate a 220-bp fragment from mαA–TAg, representing the product of Cre-mediated Stop excision at the mαA–Stop–TAg locus. The expected 220-bp fragment was indeed obtained with genomic DNA from double-transgenic mice lenses, but not with genomic DNA from control αA–Stop–TAg mouse lenses (Fig. 4*A*). Sequence analysis of amplified 220-bp cDNA fragments revealed one single *lox* site flanked by 5′ mαA promoter sequences and 3′ TAg (Fig. 4*B*). This confirms that TAg activation had occurred, as predicted, via precise site-specific deletion of the Stop sequences in the genome of differentiating lens cells. While Stop excision

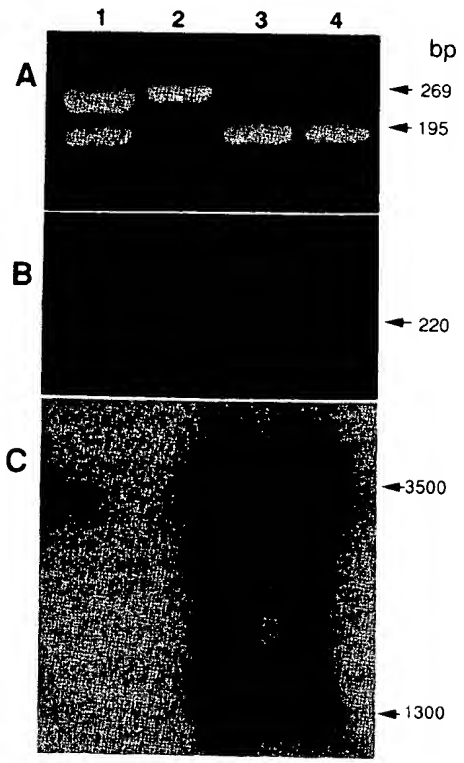


FIG. 3. Genotypes of a mαA–Stop–TAg1 × hCMV–*cre* litter. (*A*) Identification of mαA–Stop–TAg and hCMV–*cre* transgenes by PCR amplification of tail DNA. Primers for TAg generated a 195-bp fragment and primers for *cre* generated a 269-bp fragment. PCR products were separated on a 1.75% agarose gel for analysis. Lanes: 1, offspring 1; 2, offspring 2; 3, offspring 3; 4, offspring 4. Offspring 1, 3, and 4 are double transgenic. (*B*) Identification of the Stop deletion from tail genomic DNAs from the same animals. The PCR strategy selected is shown in Fig. 4*A*. Amplification yielded the expected 220-bp fragment from the three double-transgenic offspring 1, 3, and 4. (*C*) Southern hybridization analysis of *Bam*HI-digested tail DNAs from the same animals. The band of offspring 1 represents a single-copy *Bam*HI fragment of the TAg, indicating that only one mαA–TAg transgene is left in the genome, and Stop sequences (1.3 kbp) have been excised. Offspring 3 and 4 retain multiple copies of mαA–Stop–TAg transgenes in their genomes.

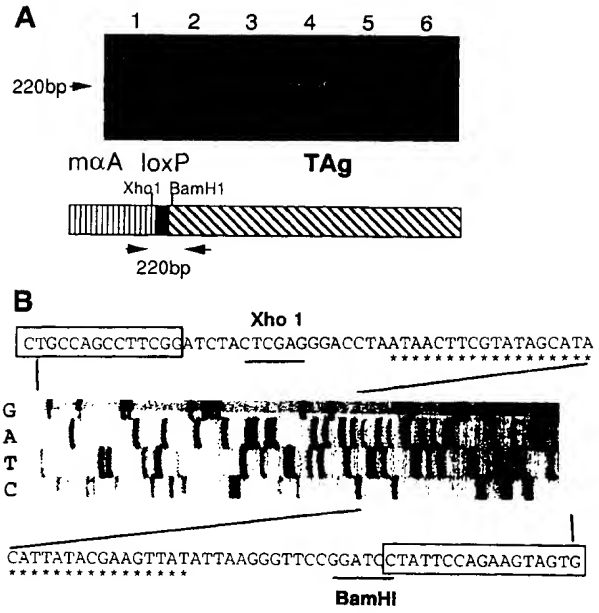


FIG. 4. PCR and sequence analysis of the *lox* regions in transgenes. (*A*) Identification of the Stop deletions from tail and eye DNAs of transgenic animals. The PCR strategy is shown below. Amplification yielded the expected 220-bp fragment from eye DNAs of all double-transgenic mice and from tail DNAs of mαA–Stop–TAg/hCMV–*cre* double-transgenic mice. PCR analysis of mαA–Stop–TAg transgenic tail DNA (lane 1) and eye DNA (lane 2), mαA–Stop–TAg/mαA–*cre* double-transgenic tail DNA (lane 3) and eye DNA (lane 4), mαA–Stop–TAg/hCMV–*cre* double-transgenic tail DNA (lane 5) and eye DNA (lane 6). (*B*) Sequence analysis of the *lox* region from the 220-bp amplification product (10). The *lox* sequence is underlined with asterisks and the mαA promoter sequences and the TAg sequences are boxed. The *Xho* I and *Bam*HI sites flanking the *lox* site are indicated.

occurred selectively in the eye lens in mice carrying the mαA–Stop–TAg and the mαA–*cre* transgenes, the constellation of mαA–Stop–TAg plus hCMV–*cre* transgenes resulted in Stop excision in other Cre-expressing tissues as well (Figs. 3*B* and 4*A*). However, the phenotypes of the two types of the double-transgenic mice did not visibly differ because TAg is driven by the mαA promoter and is therefore synthesized almost exclusively in the lens (17). Southern analysis of tail DNAs obtained from a double-transgenic litter demonstrated that the Cre expressed from the hCMV promoter is highly efficient in deleting not only the Stop sequences but the multiple copies of the mαA–Stop–TAg transgene as well, until only a single mαA–TAg with a single *lox* site is left in the genome (Fig. 3*C*).

Conclusions. Our studies have shown that the *cre/lox* system is a very powerful tool to activate a transgene by incorporating two different transgenes in one genome. In this respect, it may be compared with other binary systems that have been developed for a similar purpose (24–26). Because the Cre recombinase is a member of the Int family of recombinases (27), it is likely that other recombinases will be useful in directing precise site-specific DNA rearrangements in transgenic animals. For example, the FLP recombinase of *Saccharomyces cerevisiae* has been shown to be proficient for recombination in both *Drosophila* (28) and in cultured mammalian cells (29). The pSR1 recombinase of *Zygosaccharomyces rouxii* appears to be quite similar to the Cre and FLP recombinases and has been shown to function efficiently in *S. cerevisiae* (30).

The activation of TAg in the developing lens is likely to occur in any lens cell that produces Cre at a level sufficient to allow lox synapsis and excision of Stop. Cells activating TAg at slightly different stages of embryonic lens development may be expected to be immortalized by TAg and to give rise to foci of transformed cells that reflect the differentiated state of the initial target cell. Thus, a careful analysis of cell types found in the developing double-transgenic lens may enable us to describe more precisely the effects of tissue differentiation on the process of oncogenesis.

The amount of Cre recombinase activity produced in *cre* mice is likely to govern the efficiency with which recombination takes place. It is encouraging that the Cre recombinase in hCMV–*cre* mice is quite proficient for recombination despite the fact that it is derived from a transgene that lacks an optimal eukaryotic translation initiation signal (31, 32). In cultured cells, the change to an efficient Cre translation initiation signal for the *cre* gene results in a substantial increase in recombination ability (16). We anticipate that this enhancement would also occur in transgenic animals, thus allowing promoters of even moderate strength to be useful for driving Cre expression in binary systems.

The utility of the *cre/lox* system for transgenic technology is likely to go far beyond the application we chose for our study. Based on the findings we report here, a great many schemes can be designed aimed at modulating transgene activity precisely and efficiently *in vivo* while simultaneously reducing the transgene copy number at any given integration site, down to one. A potentially even more powerful tool for gene modulation *in vivo* is the known propensity of Cre to mediate not only excision but also integration events. Cre will insert DNA sequences at preestablished chromosomal *lox* sites in the mammalian genome (16). Such integration events are infrequent and are therefore likely to require selection in embryonic stem cells before they can be established in the mouse germ line.

We thank D. LaBarre, C. George, S. P. Huang, and W. Baubonis for expert technical assistance; L. G. Davis for his support; D. Cogan and K. Mahon for advice; and G. Dressler and G. Wong for their comments on the manuscript.

1. Craig, N. L. (1988) *Annu. Rev. Genet.* **22**, 77–105.
2. Roca, A. I. & Cox, M. M. (1990) *Crit. Rev. Biochem. Mol. Biol.* **25**, 415–456.
3. Sandmeier, H., Iida, S., Meyer, J., Hiestand-Nauer, R. & Arber, W. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1109–1113.
4. Smith, G. R. (1989) *Genome* **31**, 520–527.
5. Stark, W. M., Boocock, M. R. & Sherratt, D. J. (1989) *Trends Genet.* **5**, 304–309.
6. Araki, H., Tatsumi, H., Sakurai, T., Ushio, K., Muta, T. & Oshima, Y. (1985) *J. Mol. Biol.* **182**, 191–203.
7. Broach, J. R. & Hicks, J. B. (1980) *Cell* **21**, 501–508.
8. Oettinger, M. A., Schatz, D. G., Gorka, C. & Baltimore, D. (1990) *Science* **248**, 1517–1523.
9. Petes, T. D., Detloff, P., Jinks-Robertson, S., Judd, S. R., Kupoec, M., Nag, D., Stapleton, A., Symington, L. S., Vincent, A. & White, M. (1989) *Genome* **31**, 536–540.
10. Schatz, D. G., Oettinger, M. A. & Baltimore, D. (1989) *Cell* **59**, 1035–1048.
11. Austin, S., Ziese, M. & Sternberg, N. (1983) *Cell* **25**, 729–736.
12. Hoess, R. & Abremski, K. (1985) *J. Mol. Biol.* **181**, 351–362.
13. Sternberg, N. & Hamilton, D. (1981) *J. Mol. Biol.* **150**, 467–486.
14. Sauer, B. (1987) *Mol. Cell. Biol.* **7**, 2087–2096.
15. Sauer, B. & Henderson, N. (1989) *Nucleic Acids Res.* **17**, 147–161.
16. Sauer, B. & Henderson, N. (1990) *New Biol.* **2**, 441–449.
17. Mahon, K. A., Chepelinsky, A. B., Khillan, J. S., Overbeek, P. A., Piatigorsky, J. & Westphal, H. (1987) *Science* **235**, 1622–1628.
18. Hogan, B., Costantini, F. & Lacy, E. (1986) *Manipulating the Mouse Embryo: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
19. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular*

Cloning: A Laboratory Manual (Cold Spring Harbor Lab., Cold Spring Harbor, NY), pp. 383–389.
20. Sauer, B. & Henderson, N. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5166–5170.
21. Khillan, J. S., Oskarsson, M. K., Propst, F., Kuwabara, T., Vande Woude, G. F. & Westphal, H. (1987) *Genes Dev.* **1**, 1327–1335.
22. Nakamura, T., Mahon, K. A., Miskin, R., Dey, A., Kuwabara, T. & Westphal, H. (1989) *New Biol.* **1**, 193–204.
23. Kraft, R. (1988) *BioTechniques* **6**, 544–546.
24. Byrne, G. W. & Ruddle, F. H. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 5473–5477.
25. Khillan, J. S., Deen, K. C., Yu, S.-H., Sweet, R. W., Rosenberg, M. & Westphal, H. (1988) *Nucleic Acids Res.* **16**, 1423–1430.
26. Ornitz, D. M., Moreadith, R. W. & Leder, P. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 698–702.
27. Argos, P., Landy, A., Abremski, K., Egan, J. B., Ljungquist, E. H., Hoess, R. H., Kahn, M. L., Kalionis, B., Narayana, S. V. L., Pierson, L. S., Sternberg, N. & Leong, J. M. (1986) *EMBO J.* **5**, 433–440.
28. Golic, K. G. & Lindquist, S. (1989) *Cell* **59**, 499–509.
29. O'Gorman, S., Fox, D. T. & Wahl, G. M. (1991) *Science* **251**, 1351–1355.
30. Matsuzaki, H., Nakajima, R., Nishiyama, J., Araki, H. & Oshima, Y. (1990) *J. Bacteriol.* **172**, 610–618.
31. Kozak, M. (1986) *Cell* **44**, 283–292.
32. Kozak, M. (1987) *Nucleic Acids Res.* **15**, 8125–8148.

Generalized *lacZ* expression with the ROSA26 Cre reporter strain

Mouse strains expressing the site-specific recombinase Cre (or Flp) facilitate conditional ablation of gene function when one or several exons of the gene of interest are flanked by *loxP* (or *FRT*) sites[1]. Cre expression achieved by classic transgenesis or targeting to an appropriate locus might be tissue specific, temporally restricted or inducible[2,3]. In such experimental outlines, it is necessary to monitor Cre activity at desired time points as well as to verify that Cre was not active previously during development. Other investigators have generated transgenic[4,5] or knock-in[6] lines in which *lacZ* expression is conditional on the removal of an intervening segment. However, such lines are most useful if *lacZ* can be expressed in all cell types and hence is driven off a constitutively active promoter in the mouse.

We have previously described a gene-trap strain, ROSA βgeo 26, in which expression of the βgeo reporter appears to be constitutive during embryonic development[7,8]. I report here successful targeting at the ROSA26 locus and the derivation of a reporter line for monitoring Cre expression. To target the locus, a 5-kb genomic fragment was subcloned in a plasmid vector along with a diphtheria toxin (DTA) expression cassette for negative selection to produce the vector pROSA26-1. A splice acceptor sequence (SA) identical to the one used in the original gene-trap allele, a neo expression cassette flanked by *loxP* sites, a *lacZ* gene and a polyadenylation (bpA) sequence were inserted at a unique *Xba*I site approximately 300-bp 5′ of the original gene-trap integration site (Fig. 1*a*). A triple polyadenylation sequence[9] was added to the 3′ end of the neo expression cassette to prevent transcriptional read-through. This ROSA26 reporter (R26R) construct was linearized with *Kpn*I and electroporated into AK7 embryonic stem (ES) cells. Following G418 selection, 8 of 23 G418^r colonies were found to have correctly undergone homologous recombination by PCR and were further verified by Southern-blot analysis (Fig. 1*b*). Three clones were used to derive germline chimaeras. Heterozygous mice did not display an overt phenotype, and were bred to obtain viable and fertile homozygous mutant progeny.

The recombination efficiency will depend on the level of Cre expression and thus vary between different mouse strains. Heterozygous R26R mice were bred with R26Cre mice, a general deletor mouse line made by targeting Cre to the ROSA26 locus, and embryos were collected at various stages between embryonic day (E) 8 and E16 and stained with X-Gal for *lacZ* activity. Embryos heterozygous for both R26Cre and R26R alleles displayed ubiquitous blue staining, whereas wild-type or heterozygous R26R embryos did not show any staining (Fig. 2). These results indicate that the reporter line functions as planned and that following recombination at preimplantation, at which time the ROSA26 promoter is activated[7], *lacZ* can be expressed in all cells of the embryo. Crossing R26R mice with other Cre-expressing strains resulted in different or more restricted *lacZ* expression patterns (data not shown). The R26R mouse strain should be of wide use for monitoring Cre expression, as well as for analysing cell lineages during development, and is available from the Induced Mutant Resource of the Jackson Laboratory (stock numbers 3309 and 3310). Another reporter mouse line has been generated at the BT5 gene-trap locus using a similar approach (S.K. Michael and E.J. Robertson, pers. comm.).

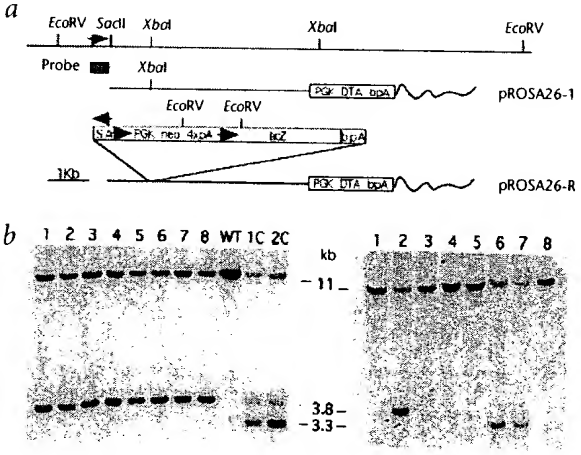


Fig. 1 ROSA 26 targeting. ***a***, Top, restriction map of the locus. PCR primers from ROSA26 flanking (5′–CCTAAAGAAGAGGCTGTGCTTTGG–3′) and splice acceptor (5′–CATCAAGGAAACCCTGGACTACTG–3′) sequences were used to amplify an approximately 1.2-kb diagnostic fragment (grey arrowheads). The probe used for Southern-blot analysis is shown as a shaded box. *LoxP* sites are indicated by black arrowheads. Only *Eco*RV sites are indicated for pROSA26-R. ***b***, Left, Southern-blot analysis of targeted clone (1–8) and wild-type (WT) DNA digested with *Eco*RV; 1C and 2C are populations of ES clones 1 and 2 transiently transfected with PGKCrebpA (a gift of M. Komada) showing the expected shorter targeted fragment due to deletion of the neo segment. Right, only the shorter *Eco*RV fragment is seen in offspring also expressing Cre (lanes 6,7) in contrast with the reporter allele alone (lane 2). ROSA26-R embryos could be genotyped by PCR (approximately 500-bp wild-type and (approximately 250-bp mutant fragments) using three oligonucleotides: 5′–AAAGTCGCTCTGAGTTGTTAT–3′, 5′–GCGAAGAGTTTGTCCTCAACC–3′ and 5′–GGAGCGGGAGAAATGGATATG–3′.

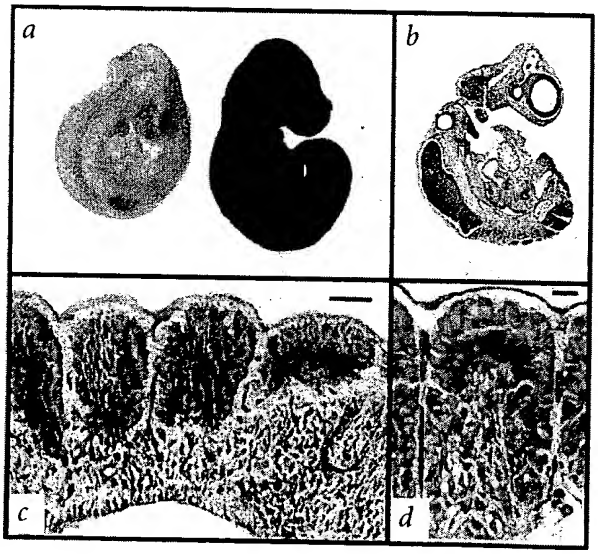


Fig. 2 *LacZ* expression following Cre recombination. ***a***, Whole-mount X-Gal staining of E9 R26R heterozygous (left) and R26R/R26Cre (right) compound heterozygous embryos. ***b***, Sagittal section of an E9 R26R/R26Cre compound heterozygous embryo. ***c***, Higher magnification (×200) sagittal section showing *lacZ* expression in all cells in the somites and the underlying mesenchyme; scale bar, 50 μm. ***d***, Cross-section through a somite (×1,000); scale bar 10 μm.

Although the ROSA26 promoter has been shown to have activity on its own and might lead to broad gene expression in transgenic mice[8], the high rate of homologous recombination and generalized *lacZ* expression observed here suggest that targeting of genes to the ROSA 26 locus may be a desirable method to achieve ubiquitous expression during development or in the adult.

Acknowledgements

I thank C. Auger, P. Capp and K. Weismann for technical assistance. This work was supported by NIH grant RO1 HD 24875.

Philippe Soriano

Program in Developmental Biology, Division of Basic Sciences, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue North, Seattle, Washington 98109, USA. e-mail: psoriano@fhcrc.org

1. Rajewsky, K. *et al. J. Clin. Invest.* **98**, 600–603 (1996).
2. Feil, R. *et al. Proc. Natl Acad. Sci. USA* **93**, 10887–10890 (1996).
3. Kellendonk, C. *et al. Nucleic Acids Res.* **24**, 1404–1411 (1996).
4. Akagi, K. *et al. Nucleic Acids Res.* **25**, 1766–1773 (1997).
5. Tsien, J.Z. *et al. Cell* **87**, 1317–1326 (1996).
6. O'Gorman, S., Dagenais, N.A., Qian, M. & Marchuk, Y. *Proc. Natl Acad. Sci. USA* **94**, 14602–14607 (1997).
7. Friedrich, G.A. & Soriano, P. *Genes Dev.* **5**, 1513–1523 (1991).
8. Zambrowicz, B.P. *et al. Proc. Natl Acad. Sci. USA* **94**, 3789–3794 (1997).
9. Maxwell, I.H., Harrison, G.S., Wood, W.M. & Maxwell, F. *BioTechniques* **7**, 276–280 (1989).

Variation in *DCP1*, encoding ACE, is associated with susceptibility to Alzheimer disease

The ε4 allele of the gene encoding apolipoprotein E (*APOE*) is the only well-replicated genetic risk factor for non-autosomal dominant forms of Alzheimer disease[1] (AD). However, the *APOE* genotype is estimated to account for less than one-half of the genetic variance[2]. Following reports that the *DCP1*D* allele of a common insertion (*I*)/deletion (*D*) polymorphism in the gene encoding angiotensin converting enzyme[3] (dipeptidyl carboxypeptidase 1; *DCP1*) is associated with increased longevity[4], we hypothesized that *DCP1*D* might protect against the development of AD and that, similarly, the *DCP1*I* allele may confer increased risk. We tested this hypothesis in a case-control sample from Cardiff and independent replication samples from London and Belfast (Table 1).

Our analysis of the Cardiff data showed a significant association between AD and *DCP1* genotypes (χ^2=8.98, d.f.=2, *P*=0.011) and alleles (χ^2=5.34, d.f.=1, *P*=0.02). The variance in genotypic distribution appeared attributable to an excess of *DCP1*I/*I* and *DCP1*I/*D* genotypes in AD cases (*P*=0.006; odds ratio (OR)=2.43, 95% CI=1.35–4.39, after Bonferroni correction for multiple testing in the present study; *P*=0.046 after correction for 8 genes studied previously in this sample). We subsequently tested this finding in the London and Belfast samples and found similar significant associations (London, *P*=0.0008, OR=2.71, 95% CI=1.5–4.9; Belfast, *P*=0.017, OR=1.82, 95% CI=1.11–2.98).

We combined the samples in a stratified analysis[5], and found that AD cases had a highly significant excess of *DCP1*I/*I* and *DCP1*I/*D* genotypes (n=542) compared with controls (n=386; χ^2=24.22, d.f.=1, *P*=0.000001). This yielded an OR of 2.22 (95% CI=1.62–3.1) with no evidence of heterogeneity between samples (*P*=0.6).

We then used logistic regression to test for the presence of the *DCP1* association after accounting for the effects of *APOE* (Table 2). After we stratified the total data set for *APOE*, the case-control differences remained highly significant and confirmed that a dominant model provides a best fit for the data (*DCP1*I/*I* and *DCP1*I/*D* versus *DCP1*D/*D*, χ^2=16.10, d.f.=1, *P*=0.00006). We observed no evidence of an interaction between alleles of *DCP1* and *APOE* (*P*=0.89). In addition, there was no evidence of association with age of onset, gender or family history (data not shown).

These findings suggest that genetic variation at the *DCP1* locus predisposes to AD in a manner that is independent of *APOE* variation. They might also help explain the unexpected association of *DCP1*D/*D* with longevity[4]. However, we must consider the possibility that the low frequency of the *DCP1*D/*D* genotype in AD may have been due to the exclusion of cases with cardiovascular disease, for which some evidence exists of an association with the *DCP1*D/*D* genotype[6]. This appears unlikely for a number of reasons. First, the impact of the *DCP1*D/*D* genotype on cardiovascular disease is controversial, relatively small and restricted to specific geographical areas and to patient subgroups with highly heterogeneous clinical mani-

Table 1 • *DCP1* genotype and allele distributions

	Genotypes			Alleles		
	II	ID	DD	I	D	$OR_{II,ID/DD}$ (95% CI)
Cardiff cases[a] (n=198)	41 (0.21)	121 (0.61)	36 (0.18)	203 (0.51)	193 (0.49)	2.43 (1.4–4.4)
Cardiff controls (n=77)	12 (0.16)	38 (0.49)	27 (0.35)	62 (0.40)	92 (0.60)	
London cases[b] (n=135)	23 (0.17)	88 (0.65)	24 (0.18)	134 (0.50)	136 (0.50)	2.71 (1.5–4.9)
London controls (n=111)	22 (0.20)	48 (0.43)	41 (0.37)	92 (0.41)	130 (0.59)	
Belfast cases (n=209)	63 (0.30)	114 (0.55)	32 (0.15)	240 (0.57)	178 (0.43)	1.82 (1.1–3)
Belfast controls (n=198)	55 (0.28)	94 (0.47)	49 (0.25)	204 (0.51)	192 (0.49)	
Total cases[c] (n=542)	127 (0.23)	323 (0.60)	92 (0.17)	577 (0.53)	507 (0.47)	2.22 (1.6–3.1)
Total controls (n=386)	89 (0.23)	180 (0.47)	117 (0.30)	358 (0.46)	414 (0.54)	

I, *DCP1*I*; D, *DCP1*D*; II, *DCP1*I/*I*; DD, *DCP1*D/*D*; ID, *DCP1*I/*D*; n, total number of individuals genotyped; CI, confidence interval. The ascertainment, diagnosis and collection of case and control groups for the three centres are detailed elsewhere[9–11]. Mean age of onset for the cases from Cardiff, London and Belfast were 70.25 y (s.d.±9.35), 82.3 y (s.d.±6.7) and 76.60 y (s.d.±6.26), respectively. Mean age of collection for the control samples were 73.46 y (s.d.±6.2), 80.8 y (s.d.±4.5) and 77.09 y (s.d.±6.42), respectively. Cases and controls in Cardiff and London were of UK origin, whereas patients and controls for the Belfast sample consisted of individuals with parents and grandparents born in Northern Ireland. All controls had a minimum mini-mental state examination score[12] of 25. *APOE* genotypes were produced as described[13]. *DCP1* genotypes were produced using established methods[14] followed by a quality control amplification step necessary in detecting under-amplified *DCP1*I* alleles[15]. [a,b,c]Significant deviation from Hardy-Weinberg equilibrium (HWE; P_a=0.002; P_b=0.0004; P_c=0.000005), as would be expected with a genotypic association with disease. Genotypes for control groups were all within HWE (Cardiff, *P*=0.78; London, *P*=0.26; Belfast, *P*=0.5; total controls, *P*=0.47). Odds ratios for each centre and for the total samples were calculated on the risk conferred by the presence of one or more copies of the *DCP1*I* allele. Standard χ^2 test was used to test for genotypic and allelic association and logistic regression to test for *DCP1* and *APOE* interactions. Combined analysis of data from the two centres used Woolf's method[5].

1: Nat Biotechnol. 2002 Nov;20(11):1124-8.

Related Articles, Links

nature biotechnology

Site-specific genomic integration produces therapeutic Factor IX levels in mice.

Olivares EC, Hollis RP, Chalberg TW, Meuse L, Kay MA, Calos MP.

Department of Genetics, Stanford University School of Medicine, Stanford, CA 94305, USA.

We used the integrase from phage phiC31 to integrate the human Factor IX (hFIX) gene permanently into specific sites in the mouse genome. A plasmid containing attB and an expression cassette for hFIX was delivered to the livers of mice by using high-pressure tail vein injection. When an integrase expression plasmid was co-injected, hFIX serum levels increased more than tenfold to approximately 4 microg/ml, similar to normal FIX levels, and remained stable throughout the more than eight months of the experiment. hFIX levels persisted after partial hepatectomy, suggesting genomic integration of the vector. Site-specific integration was proven by characterizing and quantifying genomic integration in the liver at the DNA level. Integration was documented at two pseudo-attP sites, native sequences with partial identity to attP, with one site highly predominant. This study demonstrates in vivo gene transfer in an animal by site-specific genomic integration.

PMID: 12379870 [PubMed - indexed for MEDLINE]

Display Abstract Show: 20 Sort Send to Text

1: Genesis. 2002 Jul;33(3):119-24.

Efficient in vitro and in vivo excision of floxed sequences with a high-capacity adenoviral vector expressing Cre recombinase.

Badorf M, Edenhofer F, Dries V, Kochanek S, Schiedner G.

Center for Molecular Medicine (ZMMK), University of Cologne, Cologne, Germany.

Conditional gene expression or gene disruption using Cre/loxP- or FLP/frt-based recombination systems are valuable tools for studying gene function in development and disease. Recombinant adenoviral vectors expressing Cre recombinase have been suggested as an alternative for deletion of floxed sequences. To further improve this approach we generated a high-capacity adenoviral (HC-Ad) vector expressing Cre (HC-Adcre). In this vector all vira coding sequences are deleted resulting in decreased toxicity. In the present study HC-Adcre efficiently mediated recombination between two loxP sites located in the genome of a reporter cell line. When intravenously injected int ROSA26 reporter mice, a floxed sequence was excised in hepatocytes resulting in expression of the beta-gal reporter. Our data indicate that HC-Ad vectors expressing Cre effectively delete floxed sequences in vivo and have a significant potential as a tool for functional studies in mice. Copyright 2002 Wiley-Liss, Inc.

PMID: 12124944 [PubMed - indexed for MEDLINE]

Engineering the mouse genome by site-specific recombination

Daniel Metzger* and Robert Feil†

Site-specific recombination systems are powerful tools for introducing predetermined modifications into eukaryotic genomes. Recent advances allow the manipulation of chromosomal DNA in a spatially and temporally controlled manner in mice, offering unprecedented possibilities for studying mammalian genome function and for generating animal models for human diseases.

Addresses
*Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, Collége de France, BP 163, 67404 Illkirch Cedex, CU de Strasbourg, France; e-mail: metzger@igbmc.u-strasbg.fr
†Institut für Pharmakologie und Toxikologie, Technische Universität München, Biedersteiner Straße 29, 80802 München, Germany; e-mail: feil@ipt.med.tu-muenchen.de

Current Opinion in Biotechnology 1999, 10:470–476

0958-1669/99/$ – see front matter

Abbreviations

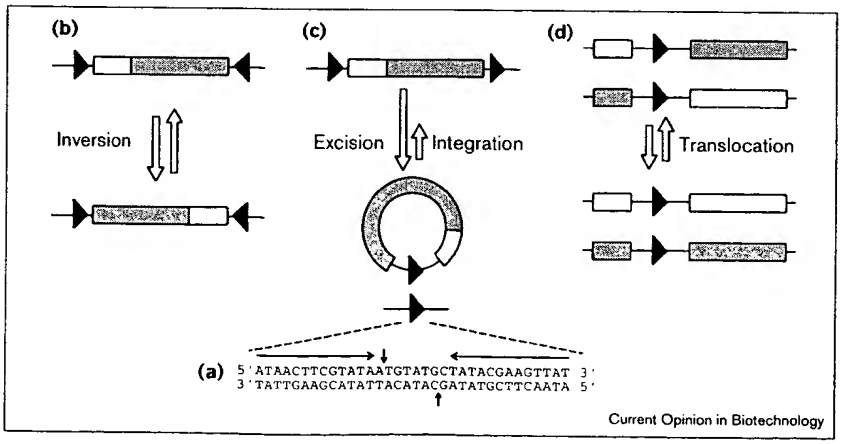
bp	basepairs
Cre	cyclization recombination
ES	embryonic stem
LBD	ligand-binding domain
loxP	locus of X-over of P1

Introduction

The ability to modify the mouse genome by random integration of transgenes or at predetermined sites by homologous recombination in embryonic stem (ES) cells has greatly advanced our understanding of mammalian gene function in health and disease [1,2]. These techniques allow one to introduce a permanent genetic alteration into the mouse germ line, but not to manipulate the genome in a spatio-temporally controlled manner *in vivo* and therefore the techniques suffer from a series of limitations. For example, a loss-of-function mutation or gene 'knockout' created by homologous recombination is present in all cells of the animal throughout pre- and postnatal development, thus precluding the analysis of the gene's function(s) in a specific cell type and at a given time. Furthermore, these methods are not suitable for engineering complex chromosomal alterations such as large deletions, duplications, inversions and translocations. To overcome these limitations, conventional genome modifications have been combined with site-specific recombination systems that rely on recombinases that promote the reciprocal exchange between two short DNA recognition sequences. Although of microbial origin, several site-specific recombination systems can function in higher eukaryotes, for example, plants, flies, and mice [3]. The Cre/*lox* system, which is highly efficient in mammalian cells, is the preferred tool for genome engineering in murine ES cells and in mice [4,5•,6].

The Cre (cyclization recombination) recombinase is a 38 kDa bacteriophage P1 protein that catalyzes recombination between two 34 bp *loxP* (locus of X-over of P1) recognition sites without the need for any cofactor. Depending on the relative orientation and location of the *loxP* sites, Cre-mediated recombination results in DNA inversion, excision/integration, and translocation, thereby confering great flexibility to genome engineering strategies (Figure 1). All reactions are reversible and, for kinetic reasons, intramolecular recombination (inversion and excision) is more efficient than intermolecular recombination (integration and translocation). Thus, it is easier to obtain stable DNA excision than stable inversion, integration and translocation. Excision of *loxP*-flanked ('floxed') DNA segments is

Figure 1



The Cre/*lox* site-specific recombination system. Cre promotes site-specific recombination between two 34 bp *loxP* recognition sequences (triangles). **(a)** The *loxP* sequence consists of two 13 bp inverted repeats (horizontal arrows) flanking an 8 bp asymmetric core region that confers an overall directionality. The two sites cleaved by Cre are indicated by vertical arrows. **(b)** Recombination between two *loxP* sites inserted into the same DNA molecule (intramolecular recombination) in opposite orientation leads to inversion of the intervening DNA segment, whereas **(c)** recombination between directly repeated *loxP* sites results in excision of the flanked DNA region (circular product) leaving one *loxP* site behind. When the *loxP* sites are located on separate DNA molecules (intermolecular recombination), **(c)** DNA integration or **(d)** translocation can be achieved.

Table 1

Examples of tissue-specific and inducible Cre transgenic mice.

Strain	Promoter	Main tissue of recombination	References
Tissue-specific Cre mice			
mαA-Cre	αA-crystallin	Eye lens	[9]
CaMKIIa-Cre	Calcium/calmodulin-dependent protein kinase IIα	Forebrain (CA1 pyramidal cells)	[67]
P0-Cre	P0 gene	Schwann cells	[68]
POMC-Cre	Pro-opiomelanocortin	Pituitary gland (intermediate lobe)	[68]
IRBP-Cre	Interphotoreceptor retinoid binding protein	Retina (photoreceptor cells)	[68]
Wnt1-Cre	wnt-1	Nervous system	[50]
En2-Cre	Engrailed-2	Nervous system	[39]
Lck-Cre	Proximal lck	T cells	[69]
CD19-Cre	CD19 (knock in)	B cells	[70]
αMHC-Cre	α myosin heavy chain	Heart (ventricular myocytes)	[71]
MLC2v-Cre	Myosin light chain 2v (knock in)	Heart (ventricular myocytes)	[35•,72]
MCK-Cre	Muscle creatine kinase	Skeletal muscle, heart	[33•,37•]
RIP-Cre	Insulin	Pancreas (β cells)	[73,74]
Alb-Cre	Albumin enhancer/promoter	Liver	[74]
WAP-Cre	Whey acidic protein	Mammary gland, brain	[75]
BLG-Cre	β-lactoglobulin	Mammary gland	[76]
aP2-Cre	Adipose protein 2	Adipose tissue	[77]
K5-Cre	Keratin 5	Skin (basal keratinocytes)	[78]
Inducible Cre mice			
Mx-Cre	Mx1 (interferon-inducible)	Widespread (liver, spleen, etc.)	[28]
CMV-tTA/tetO-Cre	tetO/cytomegalovirus minimal (tetracycline-controlled)	Muscle, skin	[29]
Tamoxifen-activated Cre mice			
CMV-CreER^T	Cytomegalovirus	Widespread (skin, kidney, etc.)	[30,51]
Wnt1-CreER^T	wnt-1	Nervous system	[50]
Eμ/SV40-CreER^T	Ig heavy chain enhancer/SV40 early minimal promoter	B cells	[52•]
RU486-activated Cre mice			
CaMKIIa-CrePR1	Calcium/calmodulin-dependent protein kinase IIα	Brain (cortex, hippocampus)	[53•]
Thy1-CrePR1	thy-1	Brain	[53•]

most widely used for *in vivo* genome modification [5•]. The basic strategy for Cre/*lox*-directed genetic engineering is to insert *loxP* sites into chromosomes by homologous recombination in ES cells or conventional transgenesis, and then to deliver Cre to recombine them as required. Recombination between *loxP* sites can be achieved in ES cells [7] or in fertilized eggs [8] by transient Cre expression, as well as in Cre transgenic mice [9,10]. By expressing Cre during early development or in germ cells the recombined product can be transmitted into the germ line. These so-called 'deleter' mice are primarily used to remove floxed marker genes and to create null alleles [4]. In this review, we will discuss the current status of the Cre/*lox* system as a tool for mammalian genome engineering and focus on recent technological advances that allow the induction of spatio-temporally controlled genetic alterations in mice.

Cre/*lox*-assisted genome engineering strategies

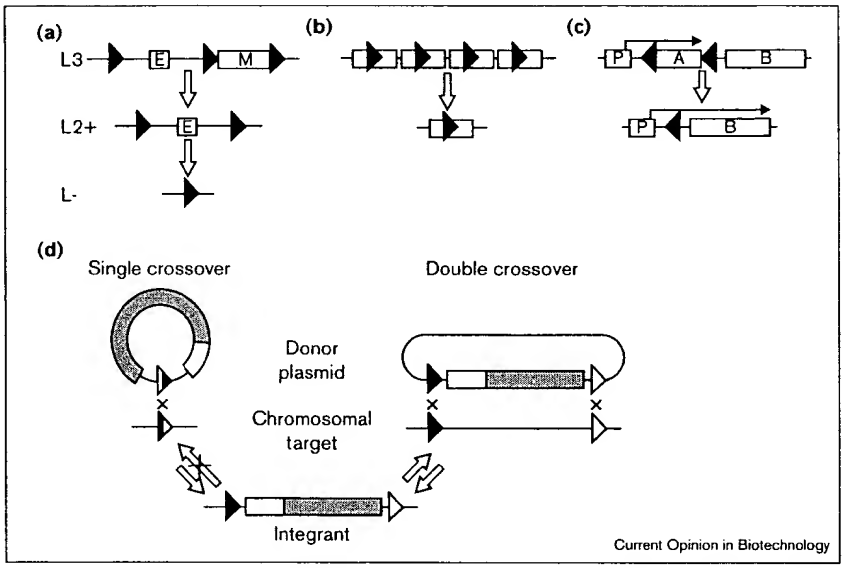
Gene inactivation

For conditional gene inactivation (Figure 2a) a floxed allele (L2+) of the target gene is created by homologous and site-specific recombination in ES cells. In general, two directly repeated *loxP* sites are inserted into intronic sequences so that they flank an essential exon and do not impair gene expression [11]. Mice carrying a conditional L2+ allele are crossed to Cre-expressing transgenic mice to excise the exon, thus creating a null allele (L–) in tissues where the recombinase is active (Figure 2a,3). The introduction of a selectable marker gene into the target locus (L3) is required to select the ES cells, but may disturb the expression of the target gene and/or nearby genes and therefore confound the analysis of the animal's phenotype [12]. Although this can be turned into an advantage by producing mutations of the target gene that downregulate its activity (hypomorphic alleles) [13,14], it is generally recommended that floxed selection cassettes are used that can be removed by Cre-mediated excision (Figure 2a).

Transgene engineering

A major drawback of conventional transgenic strategies is the lack of control over the transgene integration site and copy number, which can strongly affect transgene expression and complicate studies of gene regulatory elements in transgenic mice [15]. Strategies based on Cre-mediated transgene excision, integration, and inversion have been developed to overcome these limitations. Cre-mediated excision of *loxP*-containing transgene arrays can reduce the number of transgenes down to a single copy (Figure 2b). As the copy number can affect transgene expression, Cre-mediated transgene reduction allows the

Figure 2



Cre/*lox*-assisted genome modification strategies. **(a)** Gene inactivation. A DNA construct carrying three directly repeated *loxP* sites flanking an essential exon (E) of the target gene together with a selectable marker gene (M) is integrated into the target locus by homologous recombination in ES cells. This produces a potentially hypomorphic allele (L3) that can be manipulated by Cre-mediated recombination in ES cells and/or in mice. Selective excision of the selection cassette results in a floxed allele (L2+). Further Cre-mediated excision creates a null allele (L-). **(b)** Transgene reduction. Placing a *loxP* site into the transgene allows one to produce single copy transgenic lines by Cre-mediated excision of transgene arrays. **(c)** Transgene inactivation/activation. A promoter (P) drives transcription (horizontal arrow) of gene A and gene B before and after Cre-mediated excision, respectively. **(d)** Transgene integration. Stable integration of *lox*-containing DNA fragments into *lox*-tagged chromosomal sites is based on modified *lox* sites that prevent re-excision of the integrated DNA. Single crossover recombination between mutated *lox* sites (black/white triangles) generates *lox* sites that do not recombine with each other (black triangle and white triangle). Similarly, double crossover recombination between heterospecific *lox* sites that recombine with themselves but not with each other (black triangles and white triangles) facilitates site-specific DNA integration (the recombined donor plasmid is not shown).

production of transgenic mouse lines with graded levels of expression of the same transgene ([16,17•] and references therein). Furthermore, Cre-mediated DNA excision is a powerful tool for irreversibly switching between the expression of two transgenes (Figure 2c) [8]. Cre-mediated integration of a *loxP*-containing transgene into a *loxP*-tagged chromosomal site is an attractive alternative to homologous recombination for placing a single copy transgene at a predetermined chromosomal site, thus allowing reproducible transgene expression. As integration is readily reversed by the kinetically favorable re-excision, stable integration is inefficient (Figure 1c). Strategies based on recombination between modified *lox* sites that favor integration over excision, however, allow efficient site-specific DNA targeting in cultured mammalian cells (Figure 2d) [18–20]. Interestingly, efficient integration can also be achieved by injecting a *loxP*-containing transgene into a fertilized egg containing Cre and a chromosomal *loxP* site (M Rassoulzadegan, F Vidal, F Cuzin, abstract 95, Workshop on Conditional Genetic Technologies in the Mouse, Cold Spring Harbor, New York, 31 August–2 September 1998). Cre-mediated inversion of a transgene (Figure 1b) has been used to eliminate possible artifacts (such as integration site-dependent and copy number-dependent differences in the expression from different transgenic loci) in the study of gene regulatory elements [21] and to switch reversibly between the expression of two transgenes [22•] in mice. Some applications may be limited by the reversibility of inversion leading to a mixture of cells carrying either DNA orientation; however, the desired recombination product may be selectively trapped by using modified *lox* sites (see above).

Chromosome engineering

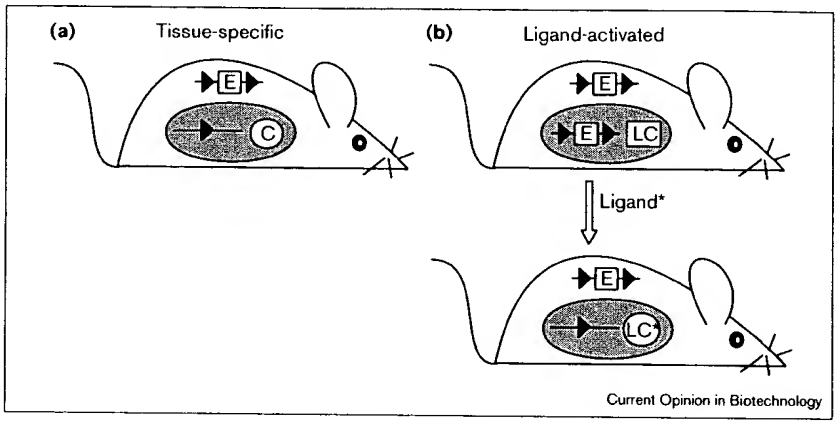
Cre/*lox*-mediated intra- and inter-chromosomal rearrangements can generate large deletions, inversions, duplications, and translocations (Figure 1). As the efficiency of intermolecular recombination is low, powerful positive selection schemes were required to construct chromosome translocations in ES cells [23–25]. Interestingly, efficient recombination between *loxP*-tagged homologous chromosomes was recently achieved using a transgenic mouse expressing Cre in spermatocytes during meiosis [26•]. As for site-specific DNA integration (see above), the efficiency of chromosome engineering may be further improved by using modified *lox* sites to trap the recombined chromosomes in the desired configuration. Note that chromosomes containing inverted *loxP* sites can be lost in Cre-expressing cells during mitosis (Cre-mediated unequal sister chromatid exchange produces an acentric chromosome that will be lost during cell division), thus producing monosomies that may confound experimental analysis [27].

Conditional somatic mutagenesis in mice

In many cases, site-specific genome modifications would be more informative if they could be selectively induced in a specific cell type and at a given time during the life of the animal. Current strategies for conditional somatic mutagenesis in mice (Figure 3) are based on cell-type-specific [9–11] or inducible [28,29] expression of Cre

Figure 3

Conditional somatic mutagenesis in mice (exemplified by conditional gene inactivation). **(a)** Tissue-specific gene inactivation is based on excision of a *loxP* (triangle)-flanked exon (E) in Cre (C)-expressing cells (shaded oval). **(b)** Temporal control over recombination can be obtained by using a ligand-dependent Cre recombinase (LC) that is inactive in the absence (boxed LC) and active in the presence (circled LC*) of a synthetic ligand (*). Spatio-temporally controlled somatic mutagenesis can be achieved by tissue-specific expression of a ligand-dependent recombinase.



using suitable promoters, as well as on ligand-dependent Cre recombinases that are selectively activated by synthetic drugs [30].

Control of Cre expression

Critical to the success of conditional somatic mutagenesis is the availability of Cre transgenic mouse strains in which Cre activity is tightly controlled in space and time. The usefulness of many Cre transgenic lines is limited by two problems inherent to general transgenic technology, namely 'leaky' and mosaic expression of the transgene. The leakiness of Cre expression from a cell-type-specific [31] or inducible [28,29,32] promoter can lead to recombination events in unwanted cell types and/or at the 'wrong' time. If the promoter is ectopically active during early embryogenesis, recombined DNA might be present in most adult tissues. On the other hand, mosaic expression of Cre prevents recombination in all cells of a given tissue and therefore complicates the analysis of a mutant phenotype. In some cases, however, mosaic genetic alterations might reproduce the pathophysiological features of sporadic genetic diseases, such as cancer.

A number of transgenic mouse lines expressing Cre in specific somatic tissues have been described (Table 1) and many of them have proven useful in addressing biological questions. Tissue-specific gene inactivation (Figure 3a) can overcome major limitations of conventional gene targeting, such as embryonic lethality and the inability to analyze whether a mutant phenotype reflects a primary/cell-autonomous requirement of the target gene in an affected cell type or arises secondary to defects in other cell types reflecting a non-cell-autonomous requirement of the target gene in the affected cells. Recent examples include the inactivation of the insulin receptor gene in skeletal muscle [33•] and in pancreatic β-cells [34•], which led to new concepts on type 2 diabetes, and the heart-specific knockout of the RXRα nuclear receptor gene [35•] and the gp130 cytokine receptor gene [36•], which revealed a non-cell-autonomous requirement for these genes in cardiomyocyte development. Furthermore, animal models for human mitochondrial DNA disorders (e.g. Kearns-Sayre syndrome) [37•] and breast cancer [38•] have been generated. Tissue-specific Cre-mediated DNA excision has also been applied to induce the expression of transgenes in mice (Figure 2c), for example, for tissue-specific activation of an oncogene [9] and for cell fate mapping studies by activation of a cellular marker gene [39]. Inducible Cre-mediated transgene inversion (Figure 1b) has been used to change the specificity of the B-cell antigen receptor in mice [22•].

Viral delivery of Cre

Spatio-temporally controlled recombination can also be achieved by the administration of Cre-encoding virus particles to mice. Tissue-specificity of recombination can be controlled by the route of virus administration, the spectrum of cells susceptible to infection, and by selection of the promoter driving Cre expression ([5•] and references therein). Adenoviral delivery of Cre was recently used to study the function of several genes in mice [40–43]; however, overall control of recombination may not be as precise as with transgenic Cre mice and viral infections may induce undesired side effects.

Ligand-activated Cre recombinases

Chimeric Cre recombinases that are selectively activated by synthetic drugs provide useful tools to control the time of recombination (Figure 3b). Based on the observation that the activity of a number of proteins can be controlled by a ligand when fused to the ligand-binding domain (LBD) of a steroid hormone receptor [44], strategies for inducible site-specific recombination have been recently developed [45,46]. Fusion of Cre to mutated LBDs of the estrogen receptor [30,47], the progesterone receptor [48], and the glucocorticoid receptor [49] results in chimeric Cre recombinases that are activated by synthetic ligands but not by natural ligands of the corresponding LBD in cultured mammalian cells. Importantly, ligand-activated site-specific recombination is feasible in mice [30] and several transgenic mouse lines have been

established that express either a tamoxifen-activated ($CreER^T$) or a RU486-activated (CrePR1) Cre recombinase (Table 1). Tamoxifen-inducible recombination can be achieved in many tissues of $CreER^T$ transgenic adult mice [30] as well as in the developing mouse embryo [50] (A Indra, P Chambon, D Metzger, unpublished data). Up to 100% recombination was observed in $CreER^T$-expressing keratinocytes [6,51] and B cells [52•] with no detectable background activity in the absence of tamoxifen. RU486-activated recombination in the brain of transgenic CrePR1 mice was less efficient, although the recombination efficiency of CrePR1 and $CreER^T$ were similar in cultured cells [53•]. These results might reflect differences in the bioavailability of RU486 and tamoxifen in various mouse tissues. Efforts are underway to further improve the efficiency of ligand-activated recombination by fusing Cre to a variety of mutated steroid receptor LBDs [49,54].

Strain validation

For proper analysis of the phenotypes produced by conditional genome modifications it is crucial to know the spatio-temporal recombination pattern of a Cre-expressing mouse at the cellular level. Monitoring of the cellular expression pattern of Cre is currently not straightforward because of the lack of antibodies allowing Cre detection in all tissues, but can be simplified by expressing a fusion protein between Cre and the green fluorescent protein [55] or an epitope-tagged Cre protein [56]. Note, however, that the absence of Cre in a given cell at a given time will not necessarily reflect a lack of recombination, which could have occurred by transient Cre expression during earlier stages. Thus, functional analysis of Cre activity is needed to properly characterize Cre transgenic mouse strains. PCR or Southern blot analysis of DNA isolated from a tissue gives an estimate of the overall recombination rate, but does not provide information about the recombination efficiency at the cellular level. Cre-mediated recombination can be monitored with single-cell resolution by using reporter mouse strains harboring a floxed cassette which, when deleted, allows the expression of a cellular marker protein, such as β-galactosidase (Figure 2c). An accurate readout of recombination is only obtained, however, if the promoter driving reporter gene expression is active in all recombined cells. Recently, three reporter strains have been described that may meet this demand [57•–59•].

Conclusions

Cre/*lox*-mediated genetic engineering has become a standard laboratory tool for functional studies of the mammalian genome [60]. Current efforts are mainly directed towards increasing the efficiency of site-specific DNA integration and large-scale chromosome manipulation, as well as refining the spatio-temporal control of recombination, a prerequisite for the establishment of sophisticated mouse models for human somatic diseases. The use of more developed transgenic technologies [61] including Cre/*lox*-assisted transgene engineering itself and the use of ligand-regulated Cre recombinases might help in obtaining reliable and tightly controlled Cre activity in transgenic mice. Without doubt, the number of useful tissue-specific and/or inducible transgenic Cre mice will rapidly increase in the future. The Jackson Laboratory (http://www.jax.org) and the European Mutant Mouse Archive (http://www.emma.rm.cnr.it) collect and distribute validated strains. A database for Cre-expressing lines has also been established (http://www.mshri.on.ca/nagy/cre.htm). Other site-specific recombinases, such as the yeast Flp recombinase [62] and the bacterial β-recombinase [63•], are also active in mammalian cells. Recent work has shown that Flp works in mice [64,65] and that mutated versions of this recombinase might have an efficiency comparable to Cre [66]. Combined use of Flp and/or β-recombinase with Cre will permit highly flexible engineering strategies, such as multiple independently controlled genetic modifications. Future applications of site-specific recombination in mice will be limited merely by the scientist's imagination and by the capacity of the animal facilities.

Acknowledgements

We apologize to all our colleagues whose studies were not cited for lack of space. We thank P Chambon and all members of his laboratory for very useful discussions as well as for communication of unpublished data, F Hofmann for continous support, and A Ammendola, M Biel and E Marais for critical reading of the manuscript.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Jaenisch R: **Transgenic animals.** *Science* 1988, **240**:1468-1474.

2. Capecchi MR: **Altering the genome by homologous recombination.** *Science* 1989, **244**:1288-1291.

3. Sauer B: **Site-specific recombination: developments and applications.** *Curr Opin Biotechnol* 1994, **5**:521-527.

4. Rajewsky K, Gu H, Kühn R, Betz UAK, Müller W, Roes J, Schwenk F: **Conditional gene targeting.** *J Clin Invest* 1996, **98**:600-603.

5. • Sauer B: **Inducible gene targeting in mice using the Cre/*lox* system.** *Methods* 1998, **14**:381-392.
A comprehensive review that provides an overview of the theoretical framework of the Cre/*lox* technology and a discussion of its various applications in mice.

6. Metzger D, Chambon P: **Site- and time-specific gene targeting in the mouse.** *Methods* 1999, in press.

7. Gu H, Zou JR, Rajewsky K: **Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-*loxP*-mediated gene targeting.** *Cell* 1993, **73**:1155-1164.

8. Araki K, Araki M, Miyazaki J, Vassalli P: **Site-specific recombination of a transgene in fertilized eggs by transient expression of Cre recombinase.** *Proc Natl Acad Sci USA* 1995, **92**:160-164.

9. Lakso M, Sauer B, Mosinger B Jr, Lee EJ, Manning RW, Yu S-H, Mulder KL, Westphal H: **Targeted oncogene activation by site-specific recombination in transgenic mice.** *Proc Natl Acad Sci USA* 1992, **89**:6232-6236.

10. Orban PC, Chui D, Marth JD: **Tissue- and site-specific DNA recombination in transgenic mice.** *Proc Natl Acad Sci USA* 1992, **89**:6861-6865.

11. Gu H, Marth JD, Orban PC, Mossmann H, Rajewsky K: **Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting.** *Science* 1994, **265**:103-106.

12. Olson EN, Arnold H-H, Rigby PWJ, Wold BJ: **Know your neighbors: three phenotypes in null mutants of the myogenic bHLH gene *MRF4*.** *Cell* 1996, **85**:1-4.

13. Meyers EN, Lewandoski M, Martin GR: **An *Fgf8* mutant allelic series generated by Cre- and Flp-mediated recombination.** *Nat Genet* 1998, **18**:136-141.

14. Nagy A, Moens C, Ivanyi E, Pawling J, Gertsenstein M, Hadjantonakis A-K, Pirity M, Rossant J: **Dissecting the role of N-*myc* in development using a single targeting vector to generate a series of alleles.** *Curr Biol* 1998, **8**:661-664.

15. Dobie K, Mehtali M, McClenaghan M, Lathe R: **Variegated gene expression in mice.** *Trends Genet* 1997, **13**:127-130.

16. de Wit T, Drabek D, Grosveld F: **Microinjection of Cre recombinase RNA induces site-specific recombination of a transgene in mouse oocytes.** *Nucleic Acids Res* 1998, **26**:676-678.

17. • Garrick D, Fiering S, Martin DIK, Whitelaw E: **Repeat-induced gene silencing in mammals.** *Nat Genet* 1998, **18**:56-59.
This study demonstrates that Cre-mediated reduction of transgene copy number can result in a marked increase in expression of a transgene in mice.

18. Araki K, Araki M, Yamamura K: **Targeted integration of DNA using mutant *lox* sites in embryonic stem cells.** *Nucleic Acids Res* 1997, **25**:868-872.

19. Bethke B, Sauer B: **Segmental genomic replacement by Cre-mediated recombination: genotoxic stress activation of the p53 promoter in single-copy transformants.** *Nucleic Acids Res* 1997, **25**:2828-2834.

20. Bouhassira EE, Westerman K, Leboulch P: **Transcriptional behavior of LCR enhancer elements integrated at the same chromosomal locus by recombinase-mediated cassette exchange.** *Blood* 1997, **90**:3332-3344.

21. Tanimoto K, Liu Q, Bungert J, Engel JD: **Effects of altered gene order or orientation of the locus control region on human β-globin gene expression in mice.** *Nature* 1999, **398**:344-348.

22. • Lam K-P, Rajewsky K: **Rapid elimination of mature autoreactive B cells demonstrated by Cre-induced change in B cell antigen receptor specificity *in vivo*.** *Proc Natl Acad Sci USA* 1998, **95**:13171-13175.
The first demonstration of inducible Cre-mediated inversion of a DNA segment in mice. This strategy can be used to study the reversibility of a mutant phenotype by switching between the expression of two transgenes.

23. Smith AJH, De Sousa MA, Kwabi-Addo B, Heppell-Parton A, Impey H, Rabbitts P: **A site-directed chromosomal translocation induced in embryonic stem cells by Cre-*loxP* recombination.** *Nat Genet* 1995, **9**:376-384.

24. van Deursen J, Fornerod M, van Rees B, Grosveld G: **Cre-mediated site-specific translocation between nonhomologous mouse chromosomes.** *Proc Natl Acad Sci USA* 1995, **92**:7376-7380.

25. Ramirez-Solis R, Liu P, Bradley A: **Chromosome engineering in mice.** *Nature* 1995, **378**:720-724.

26. • Hérault Y, Rassoulzadegan M, Cuzin F, Duboule D: **Engineering chromosomes in mice through targeted meiotic recombination (TAMERE).** *Nat Genet* 1998, **20**:381-384.
The first successful attempt to produce Cre/*lox*-directed interchromosomal rearrangements in mice. Efficient *trans*-allelic recombination of *loxP*-tagged chromosomes was achieved by using a Cre transgenic mouse strain expressing the recombinase at an early stage of male meiosis when homologous chromosome pairing occurs.

27. Lewandoski M, Martin GR: **Cre-mediated chromosome loss in mice.** *Nat Genet* 1997, **17**:223-225.

28. Kühn R, Schwenk F, Aguet M, Rajewsky K: **Inducible gene targeting in mice.** *Science* 1995, **269**:1427-1429.

29. St-Onge L, Furth PA, Gruss P: **Temporal control of the Cre recombinase in transgenic mice by a tetracycline responsive promoter.** *Nucleic Acids Res* 1996, **24**:3875-3877.

30. Feil R, Brocard J, Mascrez B, LeMeur M, Metzger D, Chambon P: **Ligand-activated site-specific recombination in mice.** *Proc Natl Acad Sci USA* 1996, **93**:10887-10890.

31. Betz UAK, Voßhenrich AJ, Rajewsky K, Müller W: **Bypass of lethality with mosaic mice generated by Cre-*loxP*-mediated recombination.** *Curr Biol* 1996, **6**:1307-1316.

32. Schmedt C, Saijo K, Niidome T, Kühn R, Aizawa S, Tarakhovsky A: **Csk controls antigen receptor-mediated development and selection of T-lineage cells.** *Nature* 1998, **394**:901-904.

33. • Brüning JC, Michael MD, Winnay JN, Hayashi T, Hörsch D, Accili D, Goodyear LJ, Kahn CR: **A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of NIDDM without altering glucose tolerance.** *Mol Cell* 1998, **2**:559-569.
See annotation to [38•].

34. • Kulkarni RN, Brüning JC, Winnay JN, Postic C, Magnuson MA, Kahn CR: **Tissue-specific knockout of the insulin receptor in pancreatic β cells creates an insulin secretory defect similar to that in type 2 diabetes.** *Cell* 1999, **96**:329-339.
See annotation to [38•].

35. • Chen J, Kubalak SW, Chien KR: **Ventricular muscle-restricted targeting of the RXRα gene reveals a non-cell-autonomous requirement in cardiac chamber morphogenesis.** *Development* 1998, **125**:1943-1949.
See annotation to [38•].

36. • Hirota H, Chen J, Betz UAK, Rajewsky K, Gu Y, Ross J Jr, Müller W, Chien KR: **Loss of a gp130 cardiac muscle cell survival pathway is a critical event in the onset of heart failure during biomechanical stress.** *Cell* 1999, **97**:189-198.
See annotation to [38•].

37. • Wang J, Wilhelmsson H, Graff C, Li H, Oldfors A, Rustin P, Brüning JC, Kahn CR, Clayton DA, Barsh GS *et al.*: **Dilated cardiomyopathy and atrioventricular conduction blocks induced by heart-specific inactivation of mitochondrial DNA gene expression.** *Nat Genet* 1999, **21**:133-137.
See annotation to [38•].

38. • Xu X, Wagner K-U, Larson D, Weaver Z, Li C, Ried T, Henninghausen L, Wynshaw-Boris A, Deng C-X: **Conditional mutation of *Brca1* in mammary epithelial cells results in blunted ductal morphogenesis and tumour formation.** *Nat Genet* 1999, **22**:37-43.
These papers [33•–38•] are recent examples demonstrating the utility of Cre/*lox*-mediated tissue-specific gene inactivation to circumvent the pre- or peri-natal lethality of a conventional gene knockout and to study the specific function of a gene in a defined set of somatic cells.

39. Zinyk DL, Mercer EH, Harris E, Anderson DJ, Joyner AL: **Fate mapping of the mouse midbrain-hindbrain constriction using a site-specific recombination system.** *Curr Biol* 1998, **8**:665-668.

40. Shibata H, Toyama K, Shioya H, Ito M, Hirota M, Hasegawa S, Matsumoto H, Takano H, Akiyama T, Toyoshima K *et al.*: **Rapid colorectal adenoma formation initiated by conditional targeting of the *apc* gene.** *Science* 1997, **278**:120-123.

41. Lee YH, Sauer B, Johnson PF, Gonzales FJ: **Disruption of the c/ebp alpha gene in adult mouse liver.** *Mol Cell Biol* 1997, **17**:6014-6022.

42. Rohlmann A, Gotthardt M, Hammer RE, Herz J: **Inducible inactivation of hepatic LRP gene by Cre-mediated recombination confirms role of LRP in clearance of chylomicron remnants.** *J Clin Invest* 1998, **101**:689-695.

43. Chang BH-J, Liao W, Li L, Nakamuta M, Mack D, Chan L: **Liver-specific inactivation of the abetalipoproteinemia gene completely abrogates very low density lipoprotein/low density lipoprotein production in a viable conditional knockout mouse.** *J Biol Chem* 1999, **274**:6051-6055.

44. Picard D: **Regulation of protein function through expression of chimaeric proteins.** *Curr Opin Biotechnol* 1994, **5**:511-515.

45. Logie C, Stewart AF: **Ligand-regulated site-specific recombination.** *Proc Natl Acad Sci USA* 1995, **92**:5940-5944.

46. Metzger D, Clifford J, Chiba H, Chambon P: **Conditional site-specific recombination in mammalian cells using a ligand-dependent chimeric Cre recombinase.** *Proc Natl Acad Sci USA* 1995, **92**:6991-6995.

47. Zhang Y, Riesterer C, Ayrall A-M, Sablitzky F, Littlewood TD, Reth M: **Inducible site-directed recombination in mouse embryonic stem cells.** *Nucleic Acids Res* 1996, **24**:543-548.

48. Kellendonk C, Tronche F, Monaghan A-P, Angrand P-O, Stewart F, Schütz G: **Regulation of Cre recombinase activity by the synthetic steroid RU 486.** *Nucleic Acids Res* 1996, **24**:1404-1411.

49. Brocard J, Feil R, Chambon P, Metzger D: **A chimeric Cre recombinase inducible by synthetic, but not by natural ligands of**

the glucocorticoid receptor. *Nucleic Acids Res* 1998, **26**:4086-4090.

50. Danielian PS, Muccino D, Rowitch DH, Michael SK, McMahon AP: **Modification of gene activity in mouse embryos *in utero* by a tamoxifen-inducible form of Cre recombinase.** *Curr Biol* 1998, **8**:1323-1326.

51. Brocard J, Warot X, Wendling O, Messaddeq N, Vonesch J, Chambon P, Metzger D: **Spatio-temporally controlled site-specific somatic mutagenesis in the mouse.** *Proc Natl Acad Sci USA* 1997, **94**:14559-14563.

52. • Schwenk F, Kühn R, Angrand P, Rajewsky K, Stewart AF: **Temporally and spatially regulated somatic mutagenesis in mice.** *Nucleic Acids Res* 1998, **26**:1427-1432.
This reference, along with [51], provides evidence that tamoxifen-activated site-specific recombination is highly efficient in mice. After administration of tamoxifen to CreER^T transgenic mice, excision of a *loxP*-flanked gene could be detected in 100% and 80% of CreER^T-expressing keratinocytes [51] and B cells [52•], respectively.

53. • Kellendonk C, Tronche F, Casanova E, Anlag K, Opherk C, Schütz G: **Inducible site-specific recombination in the brain.** *J Mol Biol* 1999, **285**:175-182.
The authors compare various ligand-dependent Cre recombinases in cultured mammalian cells and demonstrate the feasibility of ligand-activated recombination in the brain of transgenic mice expressing an RU486-activated Cre recombinase. Potential problems of ligand-activated recombination strategies and of strain validation using reporter mice are discussed.

54. Feil R, Wagner J, Metzger D, Chambon P: **Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains.** *Biochem Biophys Res Commun* 1997, **237**:752-757.

55. Gagneten S, Le Y, Miller J, Sauer B: **Brief expression of a GFP*cre* fusion gene in embryonic stem cells allows rapid retrieval of site-specific genomic deletions.** *Nucleic Acids Res* 1997, **25**:3326-3331.

56. Stricklett PK, Nelson RD, Kohan DE: **Site-specific recombination using an epitope tagged bacteriophage P1 Cre recombinase.** *Gene* 1998, **215**:415-423.

57. • Soriano P: **Generalized *lacZ* expression with the ROSA26 Cre reporter strain.** *Nat Genet* 1999, **21**:70-71.
See annotation to [59•]

58. • Lobe CG, Koop KE, Kreppner W, Lomeli H, Gertsenstein M, Nagy A: **Z/AP, a double reporter for Cre-mediated recombination.** *Dev Biol* 1999, **208**:281-292.
See annotation to [59•].

59. • Mao X, Fujiwara Y, Orkin SH: **Improved reporter strain for monitoring Cre recombinase-mediated DNA excisions in mice.** *Proc Natl Acad Sci USA* 1999, **96**:5037-5042.
These papers [57•–59•] describe reporter mouse strains in which a cellular marker gene is broadly expressed after Cre-mediated recombination, thus allowing accurate monitoring of Cre activity at the cellular level in the developing embryo and in many tissues of adult mice.

60. Torres R, Kühn R: *Laboratory protocols for conditional gene targeting.* Oxford: Oxford University Press; 1997.

61. Saez E, No D, West A, Evans RM: **Inducible gene expression in mammalian cells and transgenic mice.** *Curr Opin Biotechnol* 1997, **8**:608-616.

62. O'Gorman S, Fox DT, Wahl GM: **Recombinase-mediated gene activation and site-specific integration in mammalian cells.** *Science* 1991, **251**:1351-1355.

63. • Díaz V, Rojo F, Martínez-A C, Alonso JC, Bernad A: **The prokaryotic β-recombinase catalyzes site-specific recombination in mammalian cells.** *J Biol Chem* 1999, **274**:6634-6640.
This paper describes the bacterial β-recombinase as a new tool for recombination-assisted manipulation of eukaryotic genomes. The authors show that this site-specific recombinase promotes the deletion of chromosomal DNA flanked by directly repeated *six* recognition sites in cultured mammalian cells.

64. Dymecki SM: **Flp recombinase promotes site-specific DNA recombination in embryonic stem cells and transgenic mice.** *Proc Natl Acad Sci USA* 1996, **93**:6191-6196.

65. Vooijs M, van der Valk M, te Riele H, Berns A: **Flp-mediated tissue-specific inactivation of the retinoblastoma tumor suppressor gene in the mouse.** *Oncogene* 1998, **17**:1-12.

66. Buchholz F, Angrand P-O, Stewart AF: **Improved properties of FLP recombinase evolved by cycling mutagenesis.** *Nat Biotechnol* 1998, **16**:657-662.

67. Tsien JZ, Chen DF, Gerber D, Tom C, Mercer EH, Anderson DJ, Mayford M, Kandel ER, Tonegawa S: **Subregion- and cell type-restricted gene knockout in mouse brain.** *Cell* 1996, **87**:1317-1326.

68. Akagi K, Sandig V, Vooijs M, Van der Valk M, Giovannini M, Strauss M, Berns A: **Cre-mediated somatic site-specific recombination in mice.** *Nucleic Acids Res* 1997, **25**:1766-1773.

69. Hennet T, Hagen FK, Tabak LA, Marth JD: **T-cell-specific deletion of a polypeptide N-acetylgalactosaminyl-transferase gene by site-directed recombination.** *Proc Natl Acad Sci USA* 1995, **92**:12070-12074.

70. Rickert RC, Roes J, Rajewsky K: **B lymphocyte-specific, Cre-mediated mutagenesis in mice.** *Nucleic Acids Res* 1997, **25**:1317-1318.

71. Agah R, Frenkel PA, French BA, Michael LH, Overbeek PA, Schneider MD: **Gene recombination in postmitotic cells.** *J Clin Invest* 1997, **100**:169-179.

72. Chen J, Kubalak SW, Minamisawa S, Price RL, Becker KD, Hickey R, Ross J Jr, Chien KR: **Selective requirement of myosin light chain 2v in embryonic heart function.** *J Biol Chem* 1998, **273**:1252-1256.

73. Ray MK, Fagan SP, Moldovan S, DeMayo FJ, Brunicardi FC: **A mouse model for beta cell-specific ablation of target gene(s) using the Cre-*loxP* system.** *Biochem Biophys Res Commun* 1998, **253**:65-69.

74. Postic C, Shiota M, Niswender KD, Jetton TL, Chen Y, Moates JM, Shelton KD, Lindner J, Cherrington AD, Magnuson MA: **Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic β cell-specific gene knock-outs using Cre recombinase.** *J Biol Chem* 1999, **274**:305-315.

75. Wagner K-U, Wall R, St-Onge L, Gruss P, Wynshaw-Boris A, Garrett L, Li M, Furth PA, Hennighausen L: **Cre-mediated gene deletion in the mammary gland.** *Nucleic Acids Res* 1997, **25**:4323-4330.

76. Selbert S, Bentley DJ, Melton DW, Rannie D, Lourenco P, Watson CJ, Clarke AR: **Efficient BLG-Cre mediated gene deletion in the mammary gland.** *Transgenic Res* 1998, **7**:387-396.

77. Barlow C, Schroeder M, Lekstrom-Himes J, Kylefjord H, Deng C, Wynshaw-Boris A, Spiegelman BM, Xanthopoulos KG: **Targeted expression of Cre recombinase to adipose tissue of transgenic mice directs adipose-specific excision of *loxP*-flanked gene segments.** *Nucleic Acids Res* 1997, **25**:2543-2545.

78. Tarutani M, Itami S, Okabe M, Ikawa M, Tezuka T, Yoshikawa K, Kinoshita T, Takeda J: **Tissue-specific knockout of the mouse *Pig-a* gene reveals important roles for GPI-anchored proteins in skin development.** *Proc Natl Acad Sci USA* 1997, **94**:7400-7405.

METHODS: A Companion to Methods in Enzymology 14, 381–392 (1998)
Article No. ME980593

Inducible Gene Targeting in Mice Using the Cre/*lox* System

Brian Sauer

Laboratory of Biochemistry and Metabolism, National Institute of Diabetes, Digestive and Kidney Disease, National Institutes of Health, Building 10, Room 9N119, Bethesda, Maryland 20892-1800

E-mail: sauerb@helix.nih.gov

Molecular techniques now allow the design of precise genetic modifications in the mouse. Not only can defined nucleotide changes be engineered into the genome of the mouse, but genetic switches can be designed to target expression or ablation of any gene (for which basic molecular information is available) to any tissue at any defined time. These strategies promise to contribute substantially to an increased understanding of individual gene function in development and pathogenesis. A powerful tool, both for the design of such genetic switches and for speeding the creation of gene-modified animals, is the Cre site-specific DNA recombinase of bacteriophage P1. Precise DNA rearrangements and genetic switches can be efficiently generated in a straightforward manner using Cre recombinase. In conjunction with inducible systems for controlling Cre expression and function, these recombination-based strategies are likely to have a profound impact on developmental biology and the generation of useful animal models of human disease. © 1998 Academic Press

For nearly a century the mouse has been used for genetic analysis, with this work leading to key insights in immunology, cancer biology, and development. Over the past 20 years the adoption of molecular biological techniques for the genetic manipulation of the mouse has resulted in a vast surge of interest in using the mouse as a model system for the investigation of almost all facets of mammalian biology. Remarkably, it has now become possible to genetically alter the mouse genome with nucleotide precision. Not only does this allow the direct assessment of gene function in intact animals, it also allows the design of increasingly useful animal models of human disease.

Incorporation of exogenous DNA into the mouse genome to produce a transgenic animal can be achieved by pronuclear injection of DNA in the fertilized zygote. Transgenic animals produced by this method are generally gain-of-function mutants since the transgene is designed either to express a novel gene product or to misexpress a normal gene. Precise alteration of endogenous genes (gene targeting) is accomplished by homologous recombination in embryonic stem (ES) cells and has been used extensively to generate null or "knockout" mutations. The derivation from preimplantation embryos of murine ES cells that retain totipotency even after gene targeting has allowed the generation of intact animals harboring the desired genetic alteration. Gene-targeted ES cells are injected into blastocysts and upon colonization of the germ line allow generation of intact animals with the desired genetic alteration. Detailed descriptions of these procedures are readily available (1, 2).

More recently, strategies exploiting site-specific DNA recombination have been incorporated into transgenic and gene-targeting procedures to allow *in vivo* manipulation of DNA in ES cells or living animals. A large number of site-specific DNA recombinases have been described from bacteria and yeast, and the recombination reactions that they catalyze

1046-2023/98 $25.00

span a wide range of complexity (3). Most catalyze efficient DNA recombination at sequences of from 25 to 150 bp in length, a size sufficiently large that these recognition sequences are not expected to naturally occur in the mammalian genome. The relative simplicity and efficiency of Cre recombinase from temperate phage P1 has made it particularly useful for this purpose. Placement of recombination sites into the genome and subsequent targeted expression of recombinase have allowed the development of genetic switches that can either ablate or turn on any desired gene in transgenic or gene-modified mice.

Cre-MEDIATED RECOMBINATION

Cre is the 38-kDa product of the *cre* (cyclization recombination) gene of bacteriophage P1 (4, 5) and is a site-specific DNA recombinase of the Int family (6). Because Cre is of prokaryotic origin, gene and protein designations follow standard bacterial genetic nomenclature (7). This convention also helps to avoid potential confusion with the similarly named *CRE* (cAMP response element). Cre plays two critical roles in the life of P1: it provides a backup mechanism for cyclizing P1 DNA after infection (8, 9), and it enhances P1 plasmid stability in bacterial lysogens by resolving dimeric plasmids for increased partition fidelity at bacterial division (10).

Cre recognizes a 34-bp site on the P1 genome called *loxP* (locus of X-over of P1) and efficiently catalyzes reciprocal conservative DNA recombination between pairs of *loxP* sites (11). The *loxP* site consists of two 13-bp inverted repeats flanking an 8-bp nonpalindromic core region that gives the *loxP* site an overall directionality that, by convention, is as depicted in Fig. 1. Cre-mediated recombination between two directly repeated *loxP* sites results in excision of the DNA between them as a covalently closed circle. Cre-mediated recombination between pairs of *loxP* sites in inverted orientation will result in inversion of the intervening DNA rather than excision. Breaking and joining of DNA is confined to discrete positions within the core region and proceeds one strand at a time by way of a transient phosphotyrosine DNA–protein linkage with the enzyme. Unlike many recombinases of the Int family, no accessory host factor or DNA topological requirements are required for efficient Cre-mediated DNA recombination. These two characteristics are key features that prompted the initial determination of the suitability of Cre for genomic manipulation in eukaryotic cells (12).

Not all 34 bp are essential for efficient recombination: the first 4 bp (from left to right in Fig. 1 for the left-hand repeat) of either of the 13-bp inverted repeats can tolerate some modification with little if any loss of recombinational proficiency or fidelity (13, 14). Modified *lox* sites are helpful in the design of genetic switches (see below).

Because each of the 13-bp inverted repeats of the *loxP* site binds a single Cre monomer and because Cre acts virtually stoichiometrically, the number of functional Cre molecules required for DNA synapsis and recombination is most likely 4 per recombination event (15). This is approximately what has been observed both *in vitro* and in *Escherichia coli* (unpublished results), although higher concentrations of Cre may be required in the environment of the eukaryotic cell (16). Consideration that the prokaryotic Cre protein might not enter the eukaryotic nucleus efficiently prompted the construction of Cre fusions carrying the nuclear localization signal (NLS) of SV40 T-antigen (17). Since Cre itself carries a signal(s) that directs localization exclusively to the cell nucleus in cultured mammalian cells (18), it is unclear to what degree addition of an exogenous NLS might contribute to enhanced site-specific recombination.

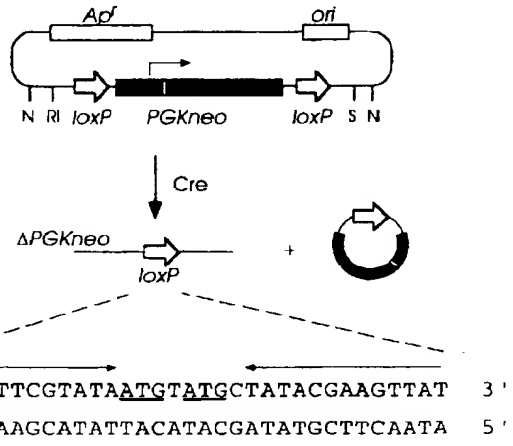


FIG. 1. Cre-mediated excisive recombination. Shown is the *lox*^{2} *neo* plasmid pBS479 in which the *PGKneo* gene from pPNT (66) was placed between the two directly repeated *loxP* sites (indicated by open arrows) of pBS246 (20). In cells carrying an integrated copy of the *lox*^{2} *neo* cassette, Cre-mediated recombination results in excision and subsequent loss of the *neo* gene. The inverted repeats of the *loxP* sequence are indicated by thin arrows, and potential ATG starts present in the orientation shown here are underlined. Restriction sites: N, *Not*I; RI, *Eco*RI; S, *Sfi*I.

TURNING GENES ON

There often arise instances where introduction of a transgene into the mouse results in either morbidity or such reduced viability that it is difficult or impossible to maintain the transgenic mouse line by breeding. In such circumstances it is advantageous to design a dormant transgene that can be activated after establishment of the transgenic line. One way of doing this is by inserting a *lox*^{2} STOP cassette (19, 20) between the (potentially toxic) transgene and its promoter (Fig. 2). After establishment of a transgenic line, the STOP signal can be removed by Cre-mediated excision, for example, by intercrossing with a second mouse expressing Cre, to activate the transgene as desired.

Lakso *et al.* (19) showed that the SV40 tumor antigen gene could be rendered completely quiescent by interposing a synthetic STOP sequence (consisting of the SV40 early polyadenylation signal, a false translational start, and a splice donor signal) between it and a lens-specific promoter. Resulting transgenic lines exhibited no incidence of tumor formation. On mating with a Cre mouse to generate doubly transgenic mice, recombinational activation of the tumor antigen gene occurred and resulted in the development of lens tumors in the offspring.

A critical issue in using the *lox*^{2} STOP strategy is that after recombination the remaining *loxP* site must not interfere with expression of the target transgene. Note that *loxP* contains two ATG translational start signals in one orientation (underlined in Fig. 1), but not in the other. Hence, it is important

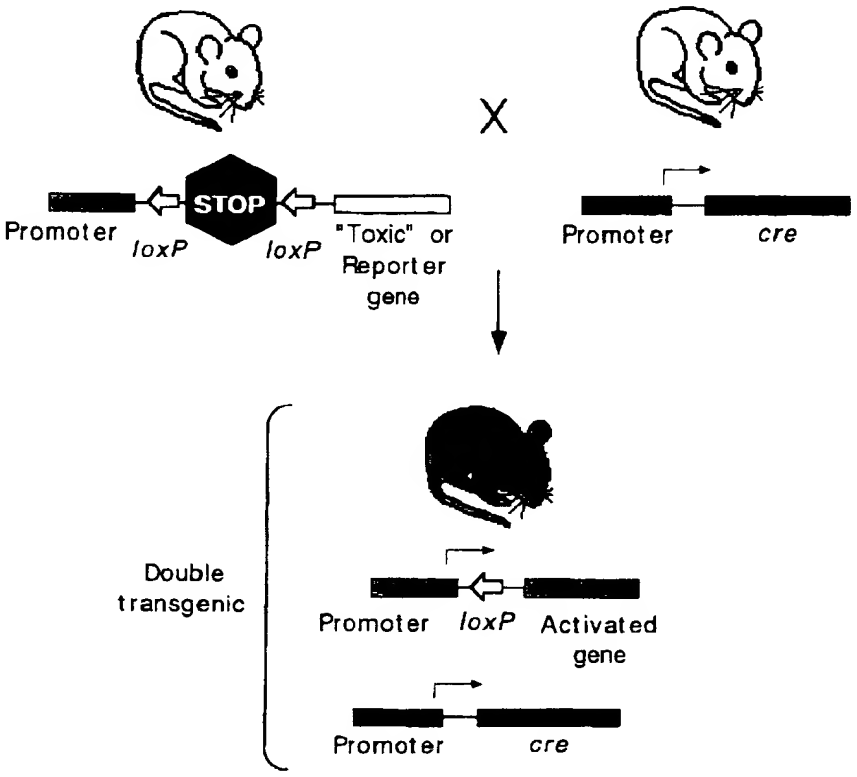


FIG. 2. Recombinational activation of gene expression. The *lox*^{2} STOP cassette from plasmid pBS302 (GenBank Accession No. U51223) is placed between the desired promoter and the "toxic" or reporter gene of interest. Mice carrying this dormant transgene are mated with a *cre* transgenic to generate a doubly transgenic mouse in which Cre has evicted the STOP sequence to activate the dormant transgene. In the example shown, the reporter transgene is capable of universal expression. Recombination by Cre occurred either ubiquitously or early after fertilization to give activation of the dormant transgene in all cells. Restriction of expression of the transgene to a particular tissue can be achieved by a suitable choice of promoters for both the reporter and the *cre* transgenes. The open arrows indicate the *loxP* site; thin arrows indicate transcription.

that the *lox*^{2} STOP cassette be oriented as shown in Fig. 2 so that there remains no false ATG start that could interfere with downstream transgene expression after excisive recombination. A second concern is that because of its palindromic structure, the *loxP* sequence will form a hairpin structure in transcribed RNA that can, if present in the 5′ leader, decrease downstream gene expression (21, 22). As discussed above, to minimize translational effects of *lox* that could affect gene expression in the design of a genetic switch, the *loxP* hairpin can be destabilized by introducing a recombinationally neutral alteration of one or more of the outer 4 bp into one of the site's inverted repeats (23).

The tissue specificity of expression for the recombinationally activated dormant transgene is a function both of the promoter specificity of the target transgene and of the promoter specificity of the Cre transgene. For example, use of a Cre transgenic mouse that expresses the recombinase ubiquitously, or that expresses Cre in the zygote, will allow activation of the dormant transgene so that the resulting expression pattern will be dictated solely by the promoter of the activated transgene. On the other hand, mating with a Cre mouse that expresses recombinase in a spatially or developmentally limited fashion will confine activation of the dormant transgene to the intersection of the two expression patterns.

Cre expression and ensuing recombination will evict the STOP sequence to allow target gene expression not only in the cell in which recombination takes place but also in its progeny. Thus the *lox*^{2} STOP strategy is a generally useful strategy both for characterizing the expression pattern of a *cre* transgene and for conducting lineage analysis in the mouse. A step in this direction is the development of a chicken β-actin promoter/*lox*^{2} STOP/*lacZ* reporter mouse that gives β-galactosidase expression in the nervous system after Cre-mediated transgene activation (24). Mating with a α-CaMKII-*cre* mouse activated expression of β-galactosidase in the CA1 cells of the cortex and hippocampus 3 weeks after birth in the doubly transgenic offspring. Note that cells that have become blue show either that Cre is being expressed in those cells or that Cre had been expressed in the lineage leading to it.

A complicating factor in these strategies is position effect variegation of the transgene (25). Position effects can lead to mosaicism of transgene expression in the target tissue and also to unexpected expression in other tissues, depending on the site of integration of the transgene. Clearly, concomitant mosaicism in expression of both the *cre* transgene and the *lox*^{2} STOP-tagged transgene could seriously hamper the attempt to activate gene expression in a majority of cells in the target tissue. For example, random mosaic expression in 60% of the target cell population for both the *cre* and the reporter transgenes would limit final reporter expression to only 36%. Acceptibility of such limitations will depend on the dictates of the particular experiment being planned. In the case of generating conditional knockout mutations (see below), mosaic *cre* expression may be a serious problem. Thus it would be prudent to screen transgenic lines for high penetrance of expression or, alternatively, to "knock-in" (26) the *cre* or reporter genes by homologous recombination in ES cells to a specific chromosomal locus displaying the desired expression pattern.

CONDITIONAL KNOCKOUTS AND MARKER EVICTION

Homologous recombination in pluripotent ES cells allows the targeted generation of a null or knockout mutation in mice. After gene targeting in ES cells, the desired mice are generated by blastocyst injection of the altered ES cells, subsequent germ line transmission of the engineered mutation, and appropriate matings to render the mutation homozygous. Because a null mutation can result in embryonic lethality that is noninformative for a gene's role either later in development or in the adult, it would be useful to devise conditional mutations of the target gene. Moreover, conditional mutations that could be confined to a particular cell lineage would greatly aid in the determination of a gene's function in that cell lineage or tissue.

Both dominant negative mutants and antisense RNA have been used to block activity of a target gene and thus can, in principle, be used to generate a null phenotype. In those situations where such strategies are likely to be effective, conditional expression of the dominant negative mutant can be attained by using the *lox*^{2} STOP strategy discussed above. After activation by mating with a *cre* transgenic with the desired tissue specificity, the dominant negative will be expressed in a manner dependent both on the pattern of Cre-mediated activation and on the promoter specificity of the activated transgene. Although this strategy may be useful for those genes for which there already exists

sufficient information to allow the design of a dominant negative mutant or a potent antisense construct, it is unlikely to be easily applicable to all genes.

An attractive alternative strategy for generating a spatially or temporally controlled conditional mutation is to modify the target gene by homologous recombination in ES cells so that it is flanked by *loxP* sites (Fig. 3). Mice containing such a modified gene are then crossed with mice expressing Cre in the desired target tissue, and Cre-mediated excision results in tissue-specific gene ablation. This strategy was first demonstrated by Gu *et al.* (27) using a mouse in which the promoter and first exon of the DNA polymerase β gene (*pol*β) were flanked by *loxP* sites. When mated to a transgenic mouse that specifically expressed Cre in T-cells, the *pol*β gene was inactivated in 40% of the T-cell population, but not in any other tissue. Incomplete elimination of the *pol*β gene from the targeted cell population in this instance may have been due to use of a wild-type *cre* transgene and most likely could be remedied by using a *cre* gene carrying a "Kozak" modification (17, 28, 29) that allows efficient translation in mammalian cells. Recombinational eradication of an endogenous gene can thus be targeted to a particular tissue or time by simply controlling expression of *cre*. Such conditional knockouts will be invaluable in obtaining a more complete understanding of gene function for genes that both play an essential role in the embryo (hence null mutations would be embryonic lethal) and provide a vital function in a particular adult tissue.

A particularly powerful feature of a conditional gene inactivation strategy using Cre is that the same *loxP*-tagged mouse can be used for gene ablation independently in a large number of different tissues, or at different developmental times, by simply mating it with a corresponding *cre* transgenic that displays the desired tissue or temporal specificity of expression. Thus, the same genetically modified animal can be used to answer a variety of different questions relating to the expression and function of the target gene. The use of the *loxP*-tagged conditional mutation strategy is doubly appealing because of the potential savings in effort and time that is necessarily associated with gene modification in ES cells and subsequent generation of an animal carrying the engineered mutation.

Conditional *loxP*-tagged mutations will also be of great value in distinguishing the relative roles of genes with apparently overlapping functions. A specific gene may be expressed in a particular adult tissue, for instance, in which there is also expressed a related gene that appears to perform a similar role. Construction of the appropriate double-gene-modified animals carrying conditional knockout mutations in these genes should allow a more accurate assessment of the relative contributions of the two genes to normal cell function in these tissues. In addition to Cre-mediated conditional loss-of-function mutations, conditional gain-of-function mutations can be designed to elucidate gene function. For example, an endogenous gene of interest can be disrupted or knocked out with the *lox*^{2} STOP cassette and then reactivated in a tissue-specific or temporally specific manner by mating with an appropriate Cre-expressing mouse.

Crucial to the success of these procedures, however, is the prior careful evaluation of the pattern of expression of the *cre* transgenic animal. Clearly, mosaic expression of recombinase in the desired tissue (or at the desired time) would defeat the intent to effect complete gene ablation in the target cell population. Conversely, unexpected expression of Cre in extraneous tissues would lead to gene ablation outside of the tissue of interest and thus complicate interpretation of the experiment. Mosaic gene expression is not uncommon in transgenic animals and is likely to be a function both of the site of integation and of the susceptibility to position effects of the promoter element. Judicious choice of a promoter displaying both position-independent gene expression and the correct combination of spatial and temporal specificity would, of course, be ideal, and some promoter/gene cassettes appear to manifest these properties. Alternatively, the use of chromatin insulator elements (30) may help in achieving these goals by ameliorating the effects of adjacent chromosomal sequences on expression of the *cre* transgene. In some cases, though, it may be preferable to obtain the desired specificity of expression by knocking-in the *cre* gene at a chromosomal locus displaying the desired pattern of expression.

The existence of both polyclonal (12) and monoclonal (31) antibodies to Cre permits Western and immunohistochemical analyses of candidate *cre* founder transgenic lines. Immunological techniques can thus be used to rapidly obtain a rough idea of the tissue-specificity of expression and to detect possible mosaicism in expression. Functional analysis of Cre activity, however, is the acid test for suitability of a particular transgenic or knock-in *cre* mouse for use in a particular conditional knockout or gene activa-

tion strategy. Because Cre-catalyzed excision is such a dramatic alteration in the genome, candidate *cre* transgenics can be mated with a reporter mouse carrying a lox^2 gene cassette, and excision can be monitored simply and quickly by performing PCR analysis of target tissues with appropriately designed

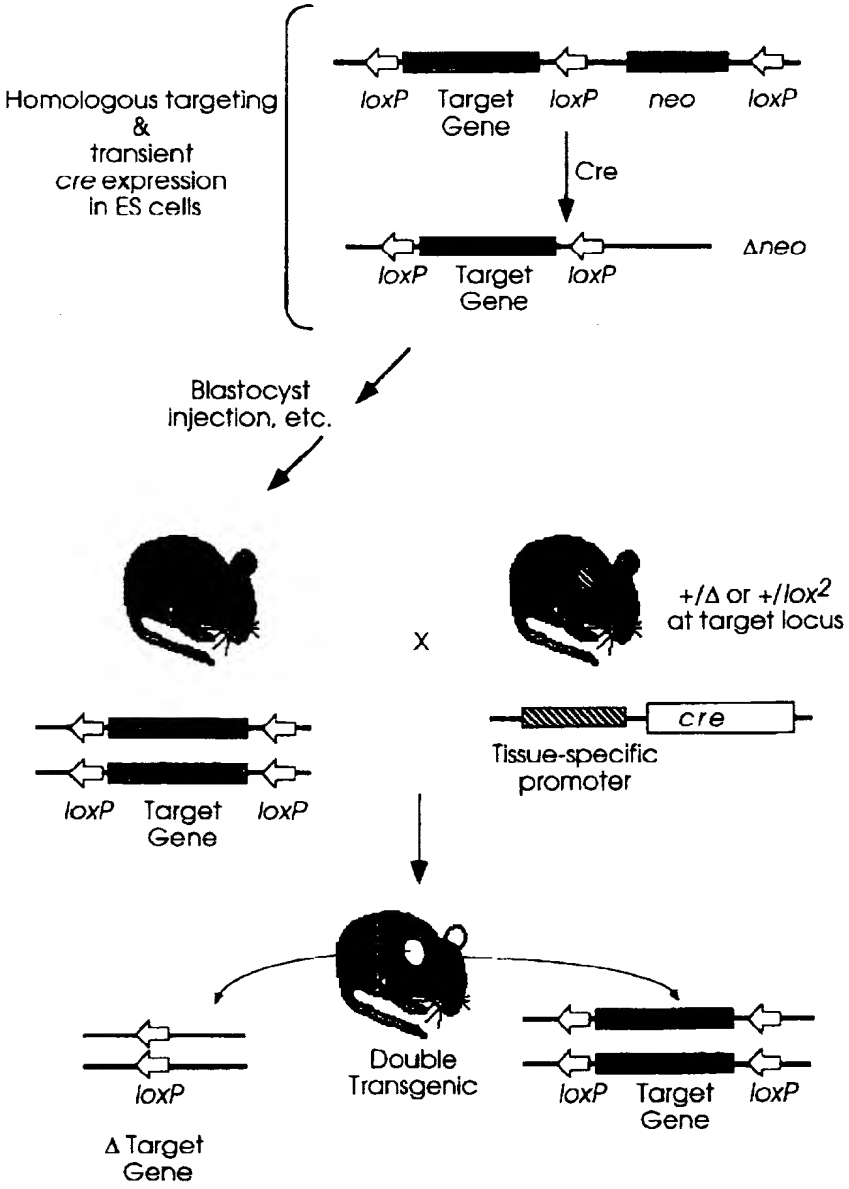


FIG. 3. Conditional gene ablation by Cre recombinase. The target endogenous gene is modified by homologous targeting in ES cells so that it is flanked by two directly repeated *loxP* sites (for simplicity, only one allele is shown). In those situations that require removal of the *neo* gene, a third *loxP* site is positioned so that *neo* can be excised by transient expression of *cre* in ES cells. The *HSVtk* gene can be incorporated into the *neo* interval to permit direct selection for Δ*neo* (27). After identification of Δ*neo* clones that retain the *loxP*-flanked target gene, mice are generated from ES cells by standard procedures. Mating of the *loxP*-modified mouse with a *cre* transgenic will generate a double-transgenic mouse in which the *loxP*-modified gene has been deleted in those tissues in which the *cre* transgene has been expressed. In the example shown, *cre* is expressed in the ears (striped) of the *cre* transgenic so that deletion of the *loxP*-modified target gene is confined solely to the ears (white) of the double transgenic.

primers. Still, the most satisfying analysis would allow a simple determination of the recombinational competence of each individual cell in the target tissue. As mentioned above, a reporter mouse carrying a *lox*^{2}STOP/*lacZ* transgene, that when activated gave good expression of *lacZ* in the nervous system, served as a powerful tool for identifying a *cre* transgenic with the desired pattern of expression in CA1 cells (24). This *cre* animal was then chosen to effect the specific ablation of a *loxP*-modified NMDA receptor gene in CA1 cells and thereby demonstrate a function for this receptor in spatial memory in the mouse (32). Reporter mice capable of expressing *lacZ* in all cells after Cre-mediated activation would be of great benefit in speeding the careful evaluation of the specificity of *cre* transgene expression, and a number of laboratories are currently developing such mice (33).

In knockout strategies, the endogenous locus in the mouse genome is targeted for *loxP* modification by homologous recombination in ES cells using a selectable marker, usually *neo*. Homologous targeting is achieved by standard procedures, with the targeting construct having a region of DNA homology that is 5′ to the target locus and another DNA segment that is homologous to the 3′ region. Sandwiched in between for conditional knockout strategies are the *loxP*-flanked region of the target gene destined for Cre-mediated excision and also the *neo* selectable marker. Optionally, the thymidine kinase gene (*tk*) of herpes simplex virus (HSV) can be appended to one of the homology arms to enhance the recovery of homologously targeted integrants using positive–negative selection (34).

Because the *neo* gene can itself adversely affect gene expression of neighboring genes (35–37), it may be important, depending on the positioning of the *neo* gene in the final targeted chromosomal locus, to be able to remove the *neo* gene from the targeted locus in a second step. Figure 3 shows that one way of achieving *neo* removal is to include a third directly repeated *loxP* site in the original targeting construct. Thus, one *loxP*-flanked interval is the gene designed for conditional inactivation, and the other *loxP*-flanked interval is *neo* itself. Complete Cre-mediated excision would, of course, remove both the *neo* gene and the targeted *loxP*-flanked DNA segment. However, transient expression of Cre in ES cells can lead to partial DNA excision, removing only the *neo* interval (27). To allow selection for the *neo* deletion, the HSV*tk* gene is included in the *neo* interval. After transient transfection with a Cre expression vector, colonies resistant to gancyclovir (TK$^-$) are screened for the desired deletion. The ratio of complete excision (target gene + *neo*) to deletion only of the *neo* interval is probably a function of the amount Cre activity introduced into the cell and thus is dependent both on the promoter used for *cre* expression and on the amount of DNA electroporated into cells. In addition, the frequency of *loxP* × *loxP* recombination may be sensitive to the distance between the sites (see below), allowing preferential excision of the *neo* interval in most cases.

Efficient eviction of the selectable marker by Cre can also simplify other genome modification procedures in ES cells. Introduction of point mutations into a gene of interest can be achieved by the strategy shown in Fig. 4, using a *lox*^{2} *neo* cassette (such as that shown in Fig. 1) for the selectable marker. After identification of those G418^R transformants in which homologous recombination has replaced one copy of the endogenous gene with the mutant variant on the targeting construct, the *neo* gene can be removed by Cre-mediated recombination, leaving behind only the 34-bp *loxP* site. Unless placed in a coding exon or another critical region of the gene, the *loxP* site is unlikely by itself to interfere with gene expression. Marker eviction by Cre also means that the selectable marker can be "recycled" for use in a second round of gene targeting (12, 38). This strategy has been used to sequentially disrupt both alleles of a target gene in ES cells with the same selectable marker (39). Marker recycling is useful because there are only a few different selectable markers that have been shown to work well in ES cells and also to allow use of a single line of drug-resistant feeder cells, since the feeder cells on which the ES cells are grown should also be resistant to the drug used for selection.

Cre catalyzes excisive recombination at chromosomal *lox* sites in 80–100% of cells into which a transient *cre* expression vector is introduced (18, 20). Hence, the major impediment to efficient *lox*^{2} marker eviction is the efficiency of DNA transfection, which can be fairly low (≤5%) in ES cells using electroporation. As mentioned above, one solution is to simply select for excision with gancyclovir by including HSV*tk* on the DNA segment to be excised. A slight drawback to this strategy is that integration of the *tk* marker into the genome precludes its use for positive–negative selection in the original targeting construct. A promising alternative strategy, which does not impose an additional round of selective stress on the cells at the marker excision step, is to

use an expression vector carrying a gene fusion of green fluorescent protein (GFP) with *cre* (Fig. 5). The GFP*cre* fusion gene readily catalyzes excisive recombination when transfected into ES cells and also results in bright green cellular fluorescence (18). By using fluorescence-activated cell sorting (FACS) of GFP*cre* transiently transfected ES cells, an enriched population can easily be obtained in which 80% or more of the cells have excised the lox^2 marker or are destined to do so. Sorted cells can then be plated for colony formation and Δ*neo* clones quickly identified for subsequent blastocyst injection. Optionally, recombinationally committed, fluorescent cells can be micromanipulated using a microscope equipped for epifluorescence (unpublished results). Parenthetically it should be noted that since knowledge of the tissue-specific pattern of Cre recombinase expression is critical for conditional gene knockout strategies, the GFP*cre* gene should also prove useful for tracing tissue-specific recombinase expression in transgenic mice.

A nagging worry in all excision strategies *in vitro* is that multiple rounds of ES cell manipulation may result in a population of cells with reduced totipotence, thus jeopardizing chances that the gene-modified locus will be transmitted through the germline. For simple marker removal, the second round of *cre* expression vector transfection of ES cells can be avoided by using instead a Cre-expressing mouse to remove the selectable marker. ES cells carrying the desired gene modification with a lox^2 *neo* gene are injected directly into blastocysts to generate a chimera, as would be done in a standard knockout strategy. The chimera is then mated with a Cre-expressing mouse to excise the *neo* gene. Both CMV-*cre* (19, 40) and EIIa-*cre* (41) mice have been found useful for giving germline excision of *neo*. Alternatively, the *neo* gene can be excised by directly injecting zygotes

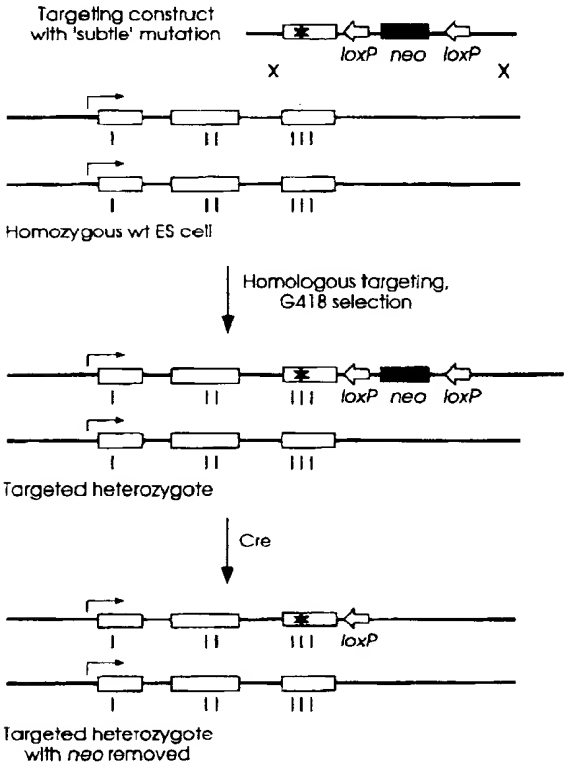


FIG. 4. Cre-mediated marker removal after introduction of a site-specific mutation. A point or other "subtle" mutation (indicated by an asterisk) is introduced into a target gene in ES cells by homologous targeting. In the hypothetical example shown here, the target gene has three exons, and the point mutation is placed into the third exon. Included on the targeting gene is a lox^2 *neo* cassette for selection, the point mutation, and flanking homology. After homologous targeting, the *neo* gene is removed by transient expression of *cre* in ES cells. Alternatively, the lox^2 *neo* ES cells can be used directly for production of mice, and the resulting chimera is mated with a Cre-expressing mouse (see text) to remove the *neo* gene. After Cre-mediated excision, only the small 34-bp *loxP* site remains in the genome.

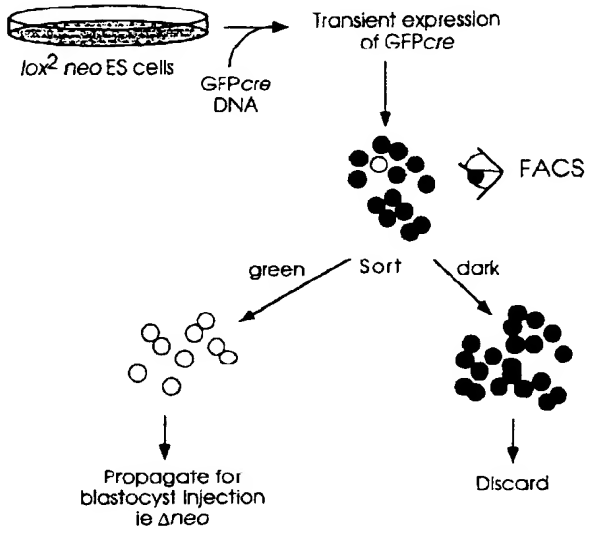


FIG. 5. Removal of *loxP*-flanked DNA in ES cells with a GFP*cre* fusion gene. ES cells carrying a *loxP*-flanked *neo* gene are transiently transfected with a vector carrying the GFP*cre* fusion gene and are FAC sorted 2 days later. Cells sorted as being (transiently) fluorescence-positive are highly enriched for cells that have undergone, or are destined to undergo, Cre-mediated marker excision.

with a DNA construct capable of expressing Cre in zygotes or early embryos (42, 43).

In the case of the EIIa-*cre* mouse, the EIIa promoter is active early in development, with high-level expression in the one-cell zygote and cessation of expression after implantation (44). The extent of excisive recombination is thereby frozen early in development and, by extension, also in the germline. Transgenic animals can thus be generated having different numbers of copies, and correspondingly different levels of expression, of a particular transgene at the same integration site. This strategy was used to demonstrate that oncogenicity in the embryonic lens from SV40 T-Ag expression could be significantly delayed by reducing the copy number of the T-Ag transgene to 1 (41).

INTERMOLECULAR RECOMBINATION AND CHROMOSOME TRANSLOCATIONS

Cre catalyzes not only intramolecular DNA recombination (excision and inversion) but also intermolecular recombination (integration and translocation). In cultured mammalian cells, this has been exploited to direct site-specific insertion of an introduced *loxP*-containing plasmid to a *loxP* target in the chromosome by cotransforming with a transient expression *cre* vector (29). Stable insertions are obtained from trapping the inserted DNA in the chromosome after a short burst of Cre activity. Although site-specific integration occurs more frequently than homologous targeting, identification of targeted integration events still requires the use of a selectable marker. Because the *loxP* target must first be placed into the genome (for example, by homologous recombination or by a more random method) it is convenient to incorporate the *loxP* site as part of a fusion gene, half of which is placed at the chromosomal target and the other half of which is on the targeting plasmid vector. Upon Cre-mediated recombination, a selectable marker gene is reconstructed, thereby permitting direct selection for the targeted chromosome insertion. A variety of *loxP* fusion markers have been generated for this purpose, including *tk* (29), *neo* (23), *hisD* (45), and, in ES cells, *Hprt* (46).

In a similar fashion, Cre can be used to orchestrate the production of precise chromosome translocations, deletions, and inversions (46–49). This is accomplished by first positioning *loxP* sites by homologous recombination in ES cells at the desired rearrangement endpoints. Each homologous targeting event for placement of the *loxP* sites consumes a selectable marker, and *neo, hph* (hygomycin resistance), and *pac* (puromycin resistance) have been used successfully in ES cells for this purpose. To facilitate subsequent selection for the desired recombination product, *Hprt*$^-$ ES cells are used for the two rounds of homologous targeting, and complementary halves of a *loxP–Hprt* fusion gene are placed at each rearrangement endpoint. Doubly targeted ES cells are then transiently transfected with a *cre* expression vector to catalyze recombination. This strategy has been used to directly select both a t(12;15) translocation between the *IgH* and the *c-myc* loci (46) and deletions and inversions at the *HoxB–wnt3* interval on chromosome 11 (48).

The Cre-mediated t(12;15) translocation was obtained at a frequency of about 5×10^{-8}. Inversions and deletions of 90 kb or 1 Mb at the *HoxB–wnt3* interval arose either at a similar frequency of about 1×10^{-7} or at a frequency of $1–5 \times 10^{-5}$, depending on the particular double-targeted ES clone. Clones giving the higher recombination frequency are those in which *loxP* sites have been targeted to the same chromosome homolog. Interestingly, the frequency of recombination in clones having *loxP* sites on different homologs was substantially lower and approached the frequency seen with translocation between chromosome heterologs. Cre also catalyzed DNA recombination between *loxP* sites located relatively far from each other in the *HoxB–wnt3* interval to generate inversions and deletions of 3–4 cM, albeit at a frequency somewhat lower than that seen for the shorter distances of 90–1000 kb.

For Cre-mediated inversions and deletions, design of the targeting *Hprt–loxP* constructs to permit Hprt$^+$ selection is straightforward if the relative orientation of the target loci is known. Correct orientation of the *loxP* sites is important to avoid the production of dicentric and acentric chromosomes after recombination. Such aberrant chromosomes would likely result in either cell inviability or loss of a normal karyotype and failure to contribute to the germline. Often, however, the relative orientation of the target loci either to each other or to the centromere is unknown. In these circumstances, it may be necessary to target each locus with both possible *Hprt–loxP* orientations (48) to ensure that a suitable double-targeted configuration is obtained.

A slightly different strategy that did not require the use of *Hprt*$^-$ ES cells was used to generate a 200-kb deletion of the amyloid precursor protein

(APP) locus (49). Two rounds of homologous recombination were used to target *loxP* sites to the desired deletion endpoints and also to position the HSV*tk* gene between the two directly oriented *loxP* sites. After transient transfection of the doubly-targeted ES cells with a *cre* expression vector, selection for resistance to FIAU resulted directly in the desired APP deletion. An intriguing alternative procedure that combined the second homologous targeting step with the Cre-mediated excision step was also presented. After placement of a *loxP* site at the first deletion endpoint along with the correctly positioned HSV*tk* gene, cells were cotransfected with the second *loxP* targeting vector and also with a *cre* expression vector. Simultaneous selection for FIAU resistance and for the dominant selectable marker on the second targeting construct (*hph*) directly gave the desired APP deletion. Although the frequency of recovery for the desired deletion was low, the substantial savings in time and effort achieved by this method is extremely attractive.

The ability to design precise inversions, deletions, and chromosome translocations in the mouse genome will have a profound impact on genetic mapping and strain construction in mice (20). In particular, many of the procedures worked out in *Drosophila* with novel chromosomes should be applicable to the mouse. Because Cre-mediated recombination is reciprocal, recombination between similarly oriented (with respect to the centromere) *loxP* sites placed at distinct loci on different chromosome homologs will result in both a deletion on one chromosome homolog and a duplication of the target interval on the other. Such balanced Cre-mediated recombination events have indeed been obtained in ES cells (48). The generation of defined segmental aneuploidies in mice will be useful not only in facilitating saturation mutagenesis of a predesignated chromosome interval, but also in evaluating the effect of increased or reduced ploidy of defined chromosome regions. This approach may be particularly valuable in identifying important chromosome regions that contribute to the many different features of trisomy 21 by allowing a systematic evaluation of increased copy number of defined chromosome 21 syntenic regions in the mouse (50; B. Sauer and M. Brennan, unpublished results).

SPECIFYING DNA RECOMBINATION IN TIME AND SPACE

Although position effects have in some instances influenced the efficiency of Cre-mediated recombination at *loxP* sites placed into the genome (51), for the most part, recombination appears to be primarily dependent only on the availability in the cell of Cre recombinase for recombination. Hence, in principle, the spatial and temporal occurrence of recombination can be completely specified by placing *cre* under the control of a promoter having the desired spatial and temporal pattern of expression. In actuality, however, some tissue-specific promoters may also be expressed at low levels in unwanted tissues or at inopportune times during development. Moreover, transgene position effects can also contribute to unexpected expression. Because even low levels of Cre expression may lead to recombination in some fraction of a cell population, use of a tightly regulated and well-characterized promoter is important in directing recombination to the correct target tissue at the correct time without unwanted recombination in nontarget tissues.

An attractive alternative to native promoters is to use synthetic inducible systems to control *cre* expression. The elegant tetracycline-regulated transcriptional systems (52, 53), presented in detail elsewhere in this issue, offer the possibility of inducing *cre* expression at a desired time either by simply dosing an animal with tetracycline or by withdrawing animals from tetracycline administration. In this strategy the *cre* gene is placed under the control of a minimal promoter (that by itself is transcriptionally silent) carrying a reiterated tet operator sequence. Transcriptional activation is achieved in cells that express a tet repressor–VP16 transcriptional activator fusion gene (tTA). Addition of tetracycline (or a tetracycline analog) prevents binding of tTA at the operator sequences to terminate gene expression. A variation is to use a mutant tet repressor–VP16 fusion that binds to operator DNA and thus activates gene expression only in the presence of tetracycline. Although mosaicism of transgene expression has limited the success of initial attempts at tetracycline control of Cre-mediated recombination in transgenic animals (54), it is likely that a judicious choice of promoter elements, or the use of knock-in strategies, will allow a robust implementation of this methodology. In a similar manner, inducible transcription of *cre* by a variety of other drugs or steroids, such as ecdysone (55, 56), RU486 (57), and dimerization-inducing synthetic ligands (58), may prove useful in mice. An intriguing second type of strategy is to control Cre function in the cell by fusing Cre to the ligand-binding domain of a steroid receptor (59–61). In cultured cells, Cre-mediated recombination by the

fusion protein is activated upon addition of ligand. The use both of steroid agonists that do not appreciably target endogenous steroid receptors and of ligand-binding domains responsive only to exogenously added ligands should allow adoption of this strategy in mice.

A complicating factor in all of these strategies is that they require multiple time-consuming mouse crosses to obtain within a single mouse the desired constellation of transgenes (for example, the regulatable *cre* gene, the transactivator gene, and the *lox*-modified target). For some purposes, an acceptable and much more rapid approach may be possible by viral-mediated gene transfer of Cre recombinase (62). Cre-expressing adenovirus vectors can be administered by intravenous injection to *loxP*-modified mice and cause recombination in the primary target tissues of liver and spleen (63, 64). Interestingly, topical application of the Cre vector to discrete regions of the brain resulted in localized recombination at the site of application (63). This is potentially a very powerful approach to achieving Cre-mediated gene ablation in a localized fashion, although it will be important to be able to distinguish the Cre-mediated gene ablation phenotype from effects due to viral infection or an associated immune response to viral infection.

CONCLUDING REMARKS

Cre DNA recombinase has become a powerful tool for the analysis of gene function in transgenic mice. Recombinational strategies in transgenic mice to turn genes on, ablate endogenous genes, and even build novel chromosomes in a tissue-specific and temporally defined manner now permit a level of genetic analysis hardly imaginable only a decade or two ago.

It is likely that further refinements and more sophisticated strategies will be developed using site-specific DNA recombination. The related recombinase FLP from *Saccharomyces cerevisiae* has also been used in transgenic mice (65), and its use in conjunction with Cre will undoubtedly permit genome manipulations that would be difficult to achieve with either recombinase alone. Moreover, the refinement of existing inducible gene expression systems and the development of new systems will allow even more precise control of recombinase expression. The combined use of these molecular tools will clearly be of considerable assistance in unraveling the complexity of mammalian development and in generating more sophisticated models of human disease.

ACKNOWLEDGMENT

I am grateful to Dr. U. Hochgeschwender for thoughtful comments on the manuscript.

REFERENCES

1. Wasserman, P. M., and DePamphilis, M. L. (1993) Guide to Techniques in Mouse Development, Academic Press, San Diego.
2. Hogan, B., Beddington, R., Constantini, F., and Lacy, E. (1994) Manipulating the Mouse Embryo: A Laboratory Annual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
3. Craig, N. L. (1988) *Annu. Rev. Genet.* **22,** 77–105.
4. Sternberg, N. (1978) *Cold Spring Harbor Symp. Quant. Biol.* **43,** 1143–1146.
5. Sternberg, N., Sauer, B., Hoess, R., and Abremski, K. (1986) *J. Mol. Biol.* **187,** 197–212.
6. Argos, P., Landy, A., Abremski, K., Egan, J. B., Ljungquist, E. H., Hoess, R. H., Kahn, M. L., Kalionis, B., Narayana, S. V. L., Pierson, L. S., Sternberg, N., and Leong, J. M. (1986) *EMBO J.* **5,** 433–440.
7. Demerec, M., Adelberg, E. A., Clark, A. J., and Hartman, P. E. (1966) *Genetics* **54,** 61–76.
8. Segev, N., and Cohen, G. (1981) *Virology* **114,** 333–342.
9. Hochman, L., Segev, N., Sternberg, N., and Cohen, G. (1983) *Virology* **131,** 11–17.
10. Austin, S., Ziese, M., and Sternberg, N. (1981) *Cell* **25,** 729–736.
11. Hoess, R. H., and Abremski, K. (1990) *in* Nucleic Acids and Molecular Biology (Eckstein, F., and Lilley, D. M. J., Eds.), Vol. 4, pp. 99–109, Springer-Verlag, Berlin/Heidelberg.
12. Sauer, B. (1987) *Mol. Cell. Biol.* **7,** 2087–2096.
13. Sauer, B., Whealy, M., Robbins, A., and Enquist, L. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 9108–9112.
14. Sauer, B. (1996) *Nucleic Acids Res.* **24,** 4608–4613.
15. Mack, A., Sauer, B., Abremski, K., and Hoess, R. (1992) *Nucleic Acids Res.* **20,** 4451–4455.
16. Sauer, B., and Henderson, N. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 5166–5170.
17. Gu, H., Zou, Y. R., and Rajewsky, K. (1993) *Cell* **73,** 1155–1164.
18. Gagneten, S., Le, Y., Miller, J., and Sauer, B. (1997) *Nucleic Acids Res.* **25,** 3326–3331.
19. Lakso, M., Sauer, B., Mosinger, J. B., Lee, E. J., Manning,

R. W., Yu, S.-H., Mulder, K. L., and Westphal, H. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 6232–6236.

20. Sauer, B. (1993) *Methods Enzymol.* **225,** 890–900.
21. Kozak, M. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 2850–2854.
22. Sauer, B., and Henderson, N. (1989) *Nucleic Acids Res.* **17,** 147–161.
23. Fukushige, S., and Sauer, B. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 7905–7909.
24. Tsien, J. Z., Chen, D. F., Gerber, D., Tom, C., Mercer, E. H., Anderson, D. J., Mayford, M., Kandel, E. R., and Tonegawa, S. (1996) *Cell* **87,** 1317–1326.
25. Lacy, E., Roberts, S., Evans, E. P., Burtenshaw, M. D., and Constantini, F. D. (1983) *Cell* **34,** 343–358.
26. Hanks, M., Wurst, W., Anson-Cartwright, L., Auerbach, A. B., and Joyner, A. L. (1995) *Science* **269,** 679–682.
27. Gu, H., Marth, J. D., Orban, P. C., Mossmann, H., and Rajewsky, K. (1994) *Science* **265,** 103–106.
28. Kozak, M. (1986) *Cell* **44,** 283–292.
29. Sauer, B., and Henderson, N. (1990) *New Biologist* **2,** 441–449.
30. Chung, J. H., Whiteley, M., and Felsenfeld, G. (1993) *Cell* **74,** 505–514.
31. Schwenk, F., Sauer, B., Kukoc, N., Hoess, R., Müller, W., Kocks, C., Kühn, R., and Rajewsky, K. (1997) *J. Immunol. Methods* **207,** 203–212.
32. Tsien, J. Z., Huerta, P. T., and Tonegawa, S. (1996) *Cell* **87,** 1327–1338.
33. Akagi, K., Sandig, V., Vooijs, M., Van der Valk, M., Giovannini, M., Strauss, M., and Berns, A. (1997) *Nucleic Acids Res.* **25,** 1766–1773.
34. Mansour, S. L., Thomas, K. R., and Capecchi, M. R. (1988) *Nature* **336,** 348–352.
35. Artelt, P., Grannemann, R., Stocking, C., Friel, J., Bartsch, J., and Hauser, H. (1991) *Gene* **99,** 249–254.
36. Rijli, F. M., Dollé, P., Fraulob, V., Lemour, M., and Chambon, P. (1994) *Dev. Dyn.* **201,** 336–377.
37. Feiring, S., Kim, C. G., Epner, E. M., and Groudine, M. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 8469–8473.
38. Sauer, B. (1994) *BioTechniques* **16,** 1086–1088.
39. Abuin, A., and Bradley, A. (1996) *Mol. Cell. Biol.* **16,** 1851–1856.
40. Schwenk, F., Baron, U., and Rajewsky, K. (1995) *Nucleic Acids Res.* **23,** 5080–5081.
41. Lakso, M., Pichel, J. G., Gorman, J. R., Sauer, B., Okamoto, Y., Lee, E., Alt, F. W., and Westphal, H. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 5860–5865.
42. Araki, K., Araki, M., Miyazaki, J.-I., and Vassalli, P. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 160–164.
43. Sunaga, S., Maki, K., Komagata, Y., Ikuta, K., and Miyazaki, J.-I. (1997) *Mol. Reprod. Dev.* **46,** 109–113.
44. Dooley, T. P., Miranda, M., Jones, N. C., and DePamphilis, M. L. (1989) *Development* **107,** 945–956.
45. Wang, P., Anton, M., Graham, F. L., and Bacchetti, S. (1995) *Somat. Cell Mol. Genet.* **21,** 429–441.
46. Smith, A. J. H., De Sousa, M. A., Kwabi-Addo, B., Heppell-Parton, A., Imprey, H., and Rabbitts, P. (1995) *Nat. Genet.* **9,** 376–385.
47. van Deursen, J., Fornerod, M., van Rees, B., and Grosveld, G. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 7376–7380.
48. Ramirez-Solis, R., Liu, P., and Bradley, A. (1995) *Nature* **378,** 720–724.
49. Li, Z.-W., Stark, G., Götz, J., Rülicke, T., Müller, U., and Weissmann, C. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 6158–6162.
50. Sauer, B. (1996) *Pharmacol. Tech.* **20,** 42–49.
51. Baubonis, W., and Sauer, B. (1993) *Nucleic Acids Res.* **21,** 2025–2029.
52. Gossen, M., and Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 5547–5551.
53. Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W., and Bujard, H. (1995) *Science* **268,** 1766–1769.
54. St-Onge, L., Furth, P. A., and Gruss, P. (1996) *Nucleic Acids Res.* **24,** 3875–3877.
55. Christopherson, K. S., Mark, M. R., Bajaj, V., and Godowski, P. J. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 6314–6318.
56. No, D., Yao, T. P., and Evans, R. M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 3346–3351.
57. Wang, Y., DeMayo, F., Tsai, S., and O'Malley, B. (1997) *Nature Biotechnol.* **15,** 239–243.
58. Ho, S. N., Biggar, S. R., Spencer, D. M., Schreiber, S. L., and Crabtree, G. R. (1996) *Nature* **382,** 822–826.
59. Metzger, D., Clifford, J., Chiba, H., and Chambon, P. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 6991–6995.
60. Kellendonk, C., Tronche, F., Monaghan, A.-P., Angrand, P.-O., and Stewart, F. (1996) *Nucleic Acids Res.* **24,** 1404–1411.
61. Zhang, Y., Riesterer, C., Ayrall, A. M., Sablitzky, F., Littlewood, T. D., and Reth, M. (1996) *Nucleic Acids Res.* **24,** 543–548.
62. Anton, M., and Graham, F. L. (1995) *J. Virol.* **69,** 4600–4606.
63. Wang, Y., Krushel, L. A., and Edelman, G. M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 3932–3936.
64. Rohlmann, A., Gotthardt, M., Willnow, T. E., Hammer, R. E., and Herz, J. (1996) *Nature Biotechnol.* **14,** 1562–1565.
65. Dymecki, S. (1996) *Proc. Natl. Acad. Sci. USA* **87,** 4712–4716.
66. Tybulewicz, V. L., Crawford, C. E., Jackson, P. K., Bronson, R. T., and Mulligan, R. C. (1991) *Cell* **65,** 1153–1163.

Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 6191–6196, June 1996
Genetics

Flp recombinase promotes site-specific DNA recombination in embryonic stem cells and transgenic mice

SUSAN M. DYMECKI

Department of Embryology, Carnegie Institution of Washington, Baltimore, MD 21210

Communicated by Allan C. Spradling, Carnegie Institution of Washington, Baltimore, MD, February 29, 1996 (received for review December 29, 1995)

ABSTRACT **Site-specific recombinases are being developed as tools for "*in vivo*" genetic engineering because they can catalyze precise excisions, integrations, inversions, or translocations of DNA between their distinct recognition target sites. Here it is demonstrated that Flp recombinase can effectively mediate site-specific excisional recombination in mouse embryonic stem cells, in differentiating embryonal carcinoma cells, and in transgenic mice. Broad Flp expression is compatible with normal development, suggesting that Flp can be used to catalyze recombination in most cell types. These properties indicate that Flp can be exploited to make prescribed alterations in the mouse genome.**

Site-specific recombinases are being developed as tools for genetic engineering because of their simplicity and precise activity in a variety of organisms. Two well-studied recombinases include Flp, from *Saccharomyces cerevisiae*, and Cre, from bacteriophage P1; both have been shown to catalyze excisions, integrations, inversions, or translocations of DNA between their distinct recognition target sites without requiring added cofactors (1–6). The type of recombination reaction is determined by the orientation of target sites relative to each other on a segment of DNA; in particular, directly repeated sites specify excision of intervening DNA.

Controlled recombinase expression in an organism carrying appropriately placed target sites can be exploited to alter the genotype of subsets of cells within an otherwise normal embryo or adult. Such mosaic animals bearing clones of genetically distinct somatic cells have been most extensively generated in *Drosophila* using Flp, providing the means to address previously intractable problems. For example, Flp-mediated excisional recombination has been used to irreversibly activate a marker gene in specific cell populations and their descendants, allowing cell lineages to be studied (7, 8); similarly, genes have been ectopically expressed to study their effects on pattern formation (9). By promoting mitotic exchange between target sites on homologous *Drosophila* chromosomes, Flp has provided an effective methodology for F_1 genetic screens (10–12). In mammalian cell culture, Flp has been shown to effectively catalyze both excision and integration of DNA at specific chromosomal sites (13–16). By catalyzing recombination between target sites on the same DNA molecule or by promoting translocations between targets sites on different DNA molecules, site-specific recombinases can be used to study a variety of biological processes. Importantly, such recombination schemes can be used to generate tissue- or stage-specific mutations that would be lethal if generated in the whole organism.

To establish some of these methods in the mouse, it may require using both homologous (gene replacement)- and site-specific recombination in embryonic stem (ES) cells to precisely place target sites in the genome. Consequently, the properties of a given recombinase should be delineated in both ES cell culture and the mouse. While Cre-mediated recombination has been successfully employed (17–21), the utility of Flp recombinase in ES cells and the mouse has not been established. Developing the technology to engineer multiple recombination reactions (independent gene activation or deletion events) using both Flp and Cre should significantly augment the tools available for molecular studies in mice. Here the utility of Flp to excise DNA in ES cells, differentiating embryonal carcinoma (EC) cells, and in transgenic mice is investigated.

MATERIALS AND METHODS

Plasmid Constructions and Production of Transgenic Mice. The *lacZ* target vector containing Flp recombinase target (FRT) sites (pFRTZ; Fig. 1*A*) was generated by inserting the *Hin*dIII/*Sal*I fragment from pSLhβAPr-lacZ-pA (22) containing human β-actin gene (h*ACTB*) sequences [3-kb 5′ flank, 78-bp 5′ untranslated region, and 832-bp first intron; ref. 23] into the unique *Hin*dIII and *Sal*I sites of pFRT$_2$neo.lacZ (24). The control plasmid pFRTZ-product was constructed by inserting the same h*ACTB* *Hin*dIII/*Sal*I fragment into pFRT.-lacZ (24). A variant of pFRTZ (designated pFRTZ.2) was generated by inserting the 1.9-kb *Xho*I/*Sal*I fragment from pIC19R-MC1TK (25) containing the herpes simplex virus thymidine kinase (HSV-tk) gene between the FRT sequences of pFRTZ. The prototype plasmid pNEOβ-GAL (ref. 13; Stratagene) was also used as target DNA. The *FLP* transgene expression vector, phACTB::FLP (Fig. 1*B*), was constructed by inserting the 3.9-kb *Xba*I/*Sal*I fragment from pSLhβAPr-lacZ-pA into the unique *Xba*I site of pFLP (24). A nonexpressing, negative control *FLP* vector (pRevhACTB::FLP) was constructed, which contains identical h*ACTB* sequences in reverse orientation. To generate pWnt1::FLP, the 2-kb *Sal*I fragment from pFLP, containing a synthetic intron, the sequence encoding Flp (ref. 13; Stratagene), and simian virus 40 early polyadenylylation (pA) sequence, was inserted into the unique *Eco*RV site of pWEXP2 (26). To produce transgenic mice, transgenes were purified away from plasmid sequences and injected into fertilized eggs from B6SJLF$_1$ × B6SJLF$_1$ mice as described (27).

Cell Culture. CCE ES cells (28) were plated onto mitomycin C-treated STO fibroblasts (29) in DMEM supplemented with 15% fetal bovine serum (FBS), 2 mM glutamine, 0.1 mM 2-mercaptoethanol, 2000 units/ml of leukemia inhibitory factor (ESGRO, GIBCO/BRL), 0.1 mM MEM nonessential amino acids, 30 μM nucleosides. Primary embryonic fibroblasts (EF) were prepared from hemizygous transgenic embryos 13.5 days post coitum as described (29). P19 EC cells

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: FRT, Flp recombination target; *FRTZ*, FRT-disrupted *lacZ* transgene; h*ACTB*; human β-actin gene; RA, retinoic acid; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; β-Gal, β-galactosidase; ES, embryonic stem; EC, embryonal carcinoma; EF, embryonic fibroblast.

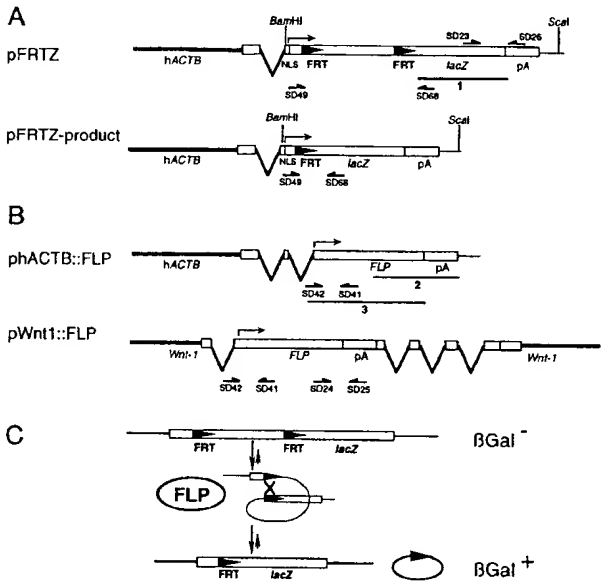


FIG. 1. DNA constructs and the Flp-mediated recombination event. (*A* and *B*) Structure of target and recombinase transgenes. FRTs are depicted as black triangles. Rectangles represent exons; heavy lines, introns and flanking regulatory sequences; thin lines, vector sequences; arrows, translation start sites. Hybridization probes are represented by numbered lines. PCR oligonucleotide primers are represented by half arrows. (*A*) Structure of target transgenes. Plasmid pFRTZ (for FRT-disrupted *lacZ* transgene) contains 3.9 kb of sequence from the human β-actin (h*ACTB*) gene (22, 23) inserted into the target vector pFRT$_2$neo.lacZ (24); a nuclear localization signal (NLS) and simian virus 40 early polyadenylylation (pA) sequence are also included. Although not shown, pFRTZ.2 is an alternative target plasmid that contains the HSV-tk gene inserted between the FRT sequences of pFRTZ and is relevant to transfections shown in Fig. 3. Control plasmid pFRTZ-product represents the product of Flp-mediated excisional recombination. Restriction sites and probe 1 used in the Southern blot analysis of Fig. 4*B* are shown on pFRTZ. (*B*) Structure of *FLP* transgenes. Plasmid phACTB::FLP contains the 3.9-kb h*ACTB* fragment inserted into the expression vector pFLP (24), which contains a synthetic intron, Flp-encoding sequence, and simian virus 40 late pA sequence from pOG44 (ref. 13; Stratagene). Although not diagrammed, pRevhACTB::FLP contains the h*ACTB* sequences in reverse orientation and serves as a negative control. Plasmid pWnt1::FLP contains the synthetic intron, Flp-encoding sequence, and the simian virus 40 late pA from pFLP inserted into the polylinker of the *Wnt-1* expression vector pWEXP2 (26). Probe 2 is relevant to the whole mount *in situ* hybridization analyses shown in Fig. 3; probe 3 is used in Northern blot analyses of Fig. 3. (*C*) Diagram of the *FLP*-mediated excisional recombination reaction.

were maintained in a 1:1 mixture of DMEM and Ham F2 medium supplemented with 7.5% FBS/2 mM glutamine.

Transient Transfections. Transient transfection of ES cells (2×10^5 ES cells in 3.5-cm dishes) was by lipofection (Lipofectamine, GIBCO/BRL) using either 0.5, 2, or 4 μg of plasmid phACTB::FLP (or negative control vector pRevhACTB::FLP) and 0.5 μg of either pFRTZ or pFRTZ-product, as indicated (Fig. 2). β-Galactosidase (β-Gal) activity was detected *in situ* using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (30). Primary EF cultures were plated (5×10^4 cells/ml) in 3.5-cm dishes and transfected by calcium phosphate precipitation (31) with 3 μg of the target pFRTZ or target pNEOβ-GAL (13) followed by X-Gal stain 48 hr later. P19 EC cells were plated (5×10^4 cells/ml) in 10-cm dishes. The next day, pairs of duplicate dishes were transfected by calcium phosphate precipitation (31) with 5 μg of target pFRTZ.2 alone or with 5 μg of phACTB::FLP or pWnt1::FLP as indicated (see Fig. 5). Twenty-four hours later one-half of the dishes were treated with either 0.5 μM of all-*trans* retinoic acid (RA; Sigma) or control diluent for an additional 5 days after which cells were stained with X-Gal.

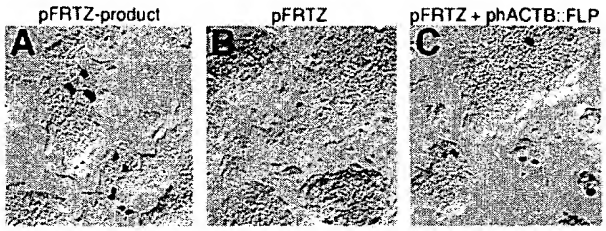


FIG. 2. Cotransfection assay for Flp function in ES cells. Flp-mediated recombination was detected by the gain of β-Gal activity as assayed by histochemical X-Gal staining (13, 30). (*A*) Positive control transfection (pFRTZ-product). ES cells were transiently transfected with 0.5 μg of pFRTZ-product plus 0.5 μg of phACTB::FLP. Although not necessary for β-Gal activity, phACTB::FLP was included to maintain equivalent amounts of h*ACTB* sequences and DNA between control and experimental transfections. (*B*) Negative control transfection (pFRTZ) included 0.5 μg of pFRTZ and 0.5 μg of the negative control Flp plasmid, pRevhACTB::FLP. (*C*) Experimental transfection (pFRTZ plus phACTB::FLP) contained 0.5 μg of pFRTZ and 0.5 μg of phACTB::FLP. To define a dose-effect relationship, ES cells were transiently transfected with 0.5 μg of pFRTZ (or pFRTZ product) and 0.5, 2, or 4 μg of phACTB::FLP. Following X-Gal staining for β-Gal expression blue-staining cells were counted. Cells staining blue after transfection with pFRTZ-product reflect transfection efficiency. The number of X-Gal-positive cells observed following transfection with pFRTZ plus phACTB::FLP were normalized to the pFRTZ product positive control values. On the basis of this estimation, 30 to 78% of the cells transfected with pFRTZ plus phACTB::FLP underwent a recombination event. Neither pFRTZ, phACTB::FLP or pRevhACTB::FLP generate β-Gal activity when transfected alone.

Transcript Detection. Whole mount *in situ* hybridization to 9.5 days post coitum embryos was performed as described (32) using single-strand digoxigenin-UTP-labeled RNA probes. The *FLP* probe (antisense probe 2, Fig. 1*B*) was a 1386-bp *Eco*RV/*Apa*I fragment from the 3′ end of the *FLP* transgene; control probe (sense) was a 648-bp *Xba*I/*Eco*RV fragment. For Northern blot analyses, fresh tissue or EF cells were homogenized in 6 M guanidinium isothiocyanate and RNA isolated using acid:phenol (33). Total cellular RNA (20 μg) was separated and assayed for hybridization to *FLP* sequence as described (34). Ethidium bromide staining of the gel and filter was used to confirm equivalent RNA loading.

Molecular Analysis of Transgenic Mouse Genotypes. Mouse tails were lysed with NaDodSO$_4$/proteinase K and treated with phenol/chloroform, 1:1 (vol/vol), precipitated with ethanol, and dissolved in 10 mM Tris·HCL, pH 8/1 mM EDTA. For PCR analysis, DNAs were amplified with the following primers: SD42 (5′-GGTCCAACTGCAGCCCAAGCTTCC-3′) and SD41 (5′-GTGGATCGATCCTACCCCTTGCG-3′), for the *FLP* transgene (a 0.75-kb amplified fragment); SD49 (5′-GACTGCTCCAAAGAAGAAGCGTAAGG-3′) and SD68 (5′-GCTATTACGCCAGCTGGCGAAAGG-3′), for the *FRTZ* transgene (a 1.4-kb amplified fragment) and FRTZ-product (a 0.25-kb fragment). The 0.25-kb PCR amplification product was cloned into plasmid pCR (TA cloning, Invitrogen) and sequenced. Genomic DNA isolated from freshly harvested tissues (35) was subjected to *Bam*HI/*Sca*I digestion, and Southern blot analyses. Radiolabeled DNA fragments (specific activity of 2–5 $\times 10^8$ cpm/μg) for use as probes were prepared by random priming (36). Transgene copy number was estimated by including standard amounts of the injected transgene in parallel. Quantitation of radioactivity in specific bands was performed with a Molecular Dynamics PhosphorImager.

RESULTS

Strategy Used to Assay Flp Function in Cell Culture and the Mouse. To generate a test recombination substrate for Flp

function, a *lacZ* gene was disrupted by inserting an FRT cassette that contains stop codons in all three reading frames (24). This target transgene is referred to as *FRTZ*, for FRT-disrupted *lacZ* (Fig. 1*A*). Because the two FRT sequences flanking the cassette are in the same orientation, Flp activity should excise the intervening DNA leaving a single residual FRT in-frame with *lacZ* (Fig. 1*C*). Because there are no ATG codons to initiate translation of functional β-Gal downstream of the FRT cassette, β-Gal activity is strictly dependent on Flp-mediated excisional recombination in a manner similar to previously described β-Gal gain-of-function systems (9, 13).

To broadly express both *FLP* and *FRTZ*, both transgenes were placed under the control of regulatory sequences from h*ACTB*) gene (Fig. 1 *A* and *B*). These h*ACTB* sequences have been shown to be active in most tissues in transgenic mice (22). A "recombined" control transgene, *FRTZ-product*, representing the predicted product of Flp recombination was also constructed (Fig. 1*A*).

Flp-Mediates Efficient Recombination of Extrachromosomal DNA in ES Cells. The efficacy of Flp-mediated excisional recombination in ES cells was tested by assaying for gain of β-Gal activity following cotransfection with target and recombinase plasmids. Cells were transiently transfected with either pFRTZ plus phACTB::FLP, or pFRTZ plus the negative control plasmid pRevhACTB::FLP, followed by X-Gal stain 48 hr later. Positive control cultures were transfected with the "recombined" plasmid, pFRTZ-product (Fig. 2*A*). Cultures transfected with target plasmid pFRTZ, alone or with pRevhACTB::FLP, showed no detectable β-Gal activity (Fig. 2*B*); in contrast, robust activity was observed following cotransfection with phACTB::FLP (Fig. 2*C*).

To estimate recombinase activity, X-Gal-positive cells in each transfection were counted and compared. The number of cells staining blue after transfection with the control "recombined" pFRTZ-product reflected transfection efficiency and, because constitutively active, the maximal number of β-Gal-positive cells. Cotransfection with a fixed amount of target plasmid and increasing amounts of *FLP* expression vector resulted in an increasing percentage of X-Gal-positive cells relative to control pFRTZ-product transfections. A comparison between experimental (pFRTZ plus phACTB::FLP) and control (pFRTZ-product) transfections showed that Flp-mediated β-Gal activation occurred in at least 30% of transfected ES cells and could be as high as 78%. This increase in recombination with increasing Flp-encoding plasmid likely reflects more Flp protein produced per cell, as well as an increase in the proportion of cells that took up both the target and Flp-encoding plasmids (and thereby had the potential to activate *lacZ*).

Flp Can Be Generally Expressed in the Mouse Without Deleterious Effects. To determine whether Flp can function in the mouse and whether Flp expression, itself, would have any adverse effects, mice carrying the h*ACTB*::*FLP* transgene were generated. To identify mouse lines producing Flp in a wide range of tissues, F_1 mice from each founder were screened for ubiquitous *FLP* mRNA and recombinase activity. The distribution and amount of *FLP* mRNA was assessed in the embryo by whole mount *in situ* hybridization and in adult tissues by Northern blot analysis. Two of the five h*ACTB*::*FLP* mouse lines exhibited broad patterns of *FLP* transcripts in 9.5 days post coitum hemizygous embryos (mouse lines 4917 and 4924; Fig. 3 *B* and *D*) and in adult tissues (Fig. 3 *E* and *F*). Flp activity was assayed in EF cultures derived from each transgenic mouse line. The EF cultures were transiently transfected with target plasmid and stained with X-Gal. Maximal Flp activity (approximately 45% of the "recombined" control) was observed in lines 4917 and 4924 (Fig. 3*G*), the same mouse lines that showed broad *FLP* expression (Fig. 3 *B* and *D*). As shown in Fig. 3 *G* and *H*, the amount of recombinase activity detected in EF cultures also correlated with the amount of *FLP* mRNA isolated from each culture. From these experiments it can be inferred that mouse lines 4917 and 4924 are the best candidates for broadly expressed active recombinase. Because no abnormalities were detected in founders or offspring it is likely that Flp activity is nontoxic and can be used in most cell types.

Flp Is Necessary and Sufficient to Recombine Target Sequences in Transgenic Mice. To test whether Flp activity can recombine a chromosomal target *in vivo*, mice carrying *FRTZ* were generated. Five transgenic founders were obtained. F_1 mice from four of the five founders bred as expected for unique single-site integration events (one founder failed to transmit the transgene). Southern blot analysis of liver DNA isolated from each mouse line showed that three of the four mouse lines carried the target *FRTZ* in head-to-tail array: line 4999 carried an array of approximately 4 copies of the *FRTZ* transgene; line 4998, 11 copies; line 5000, 30 copies. Transgene transmission was Mendelian and no rearrangements were observed.

The ability of Flp to catalyze *in vivo* recombination of the target *FRTZ* transgene was initially examined by crossing these mouse lines with the Flp producing lines described above (4917 and 4924). Tail DNA from doubly transgenic animals was analyzed by PCR using primers (diagrammed in Fig. 1 *A* and *B*) specific for detecting either the *FRTZ* transgene, the recombined target *FRTZ-product*, or the *FLP* transgene. Analyses of progeny from three distinct crosses are shown in Fig. 4*A*. The product of Flp-mediated excisional recombination at the *FRTZ* locus, was amplified only in DNA isolated from doubly transgenic mice and was not detected in littermates transgenic for only the recombinase or the target gene. All three *FRTZ* target lines were found to be competent for recombination by this assay. Sequence analysis of the 0.25-kb amplification product showed precise site-specific recombination.

Flp Mediates Recombination in a Variety of Tissues in a Dose-Dependent Manner. The efficiency of Flp recombination at target *FRTZ* loci was assayed by Southern blot analysis. Genomic DNA isolated from doubly transgenic adult mice (target line *FRTZ-4999*; *FLP-4917*) was hybridized with a *lacZ* probe (probe 1, Fig. 1*A*) to allow simultaneous detection of the target *FRTZ* transgene and the product of recombination. As shown in Fig. 4*B*, the new 4.4-kb DNA fragment resulting from the recombined target was present only in samples from doubly transgenic animals, and absent in DNA isolated from either target *FRTZ* (Fig. 4*B*) or *FLP* littermates (data not shown).

The amount of recombination product detected by Southern blot analysis was found to correlate directly with the amount of *FLP* mRNA detected in each tissue by Northern blot hybridization (Fig. 3*E*: lane 6, liver; lane 12, muscle; lane 1, testes). Estimates of recombination efficiency were obtained from phosphorimage quantification of recombined (4.4 kb) to nonrecombined (5.6 kb) bands. In muscle, approximately 30% of the transgenes were in the recombined (4.4 kb) configuration. This represents an average of the actual recombination achieved in the various cell types isolated when dissecting muscle tissue (myofibrils, connective tissue fibroblasts, vascular endothelial cells, lymph node cells, blood cells). The value of 30% therefore represents a low estimate of the maximal recombination efficiency. This frequency is consistent with that observed in the EF cell culture assay derived from the same FLP-4917 mouse line (45%, Fig. 3*G*); indeed, both cell populations showed similar amounts of *FLP* mRNA. Hybridizing with a probe specific to DNA between the FRT sites detected only the unrecombined fragment (data not shown).

A Recombined Transgene Is Stably Transmitted Through the Germ Line. A prerequisite to using Flp to genetically manipulate cell lineages is that the recombination product be stable and heritable. Germ-line transmission of the recombined transgene was demonstrated by outcrossing a doubly transgenic (*FRTZ-5000*; *FLP-4917*) male and genotyping progeny by PCR (data not shown). Both recombined and unre-

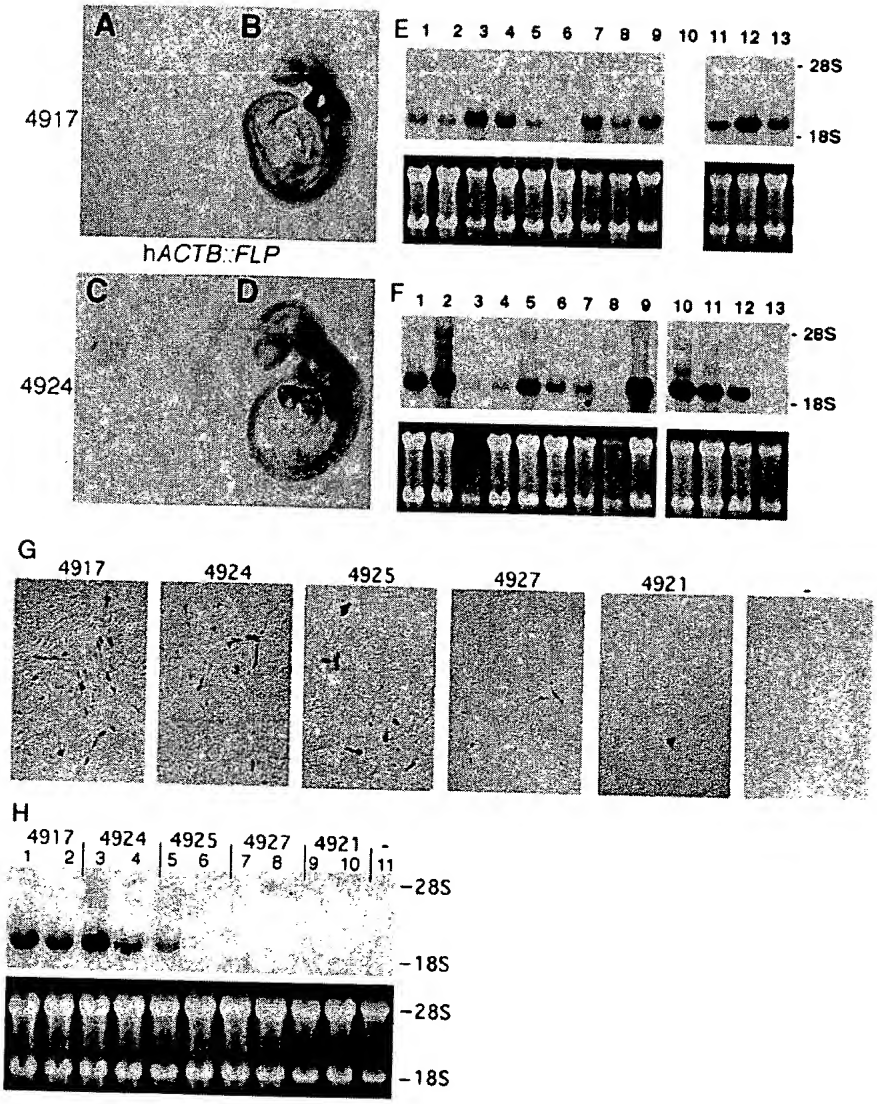


FIG. 3. Human β-actin sequences direct broad expression of *FLP* in the embryo and adult mouse without adverse affects. (*A–D*) Whole mount *in situ* hybridization analysis of *FLP* RNA expression at 9.5 days post coitum. Lateral views of nontransgenic (*A*) and transgenic (*B–D*) embryos from h*ACTB::FLP* mouse lines 4917 (*B*) and 4924 (*C* and *D*). (*A*, *B*, and *D*) *FLP* RNA detected using antisense probe 2; (*C*) control sense probe. (*E* and *F*) RNA blot analyses of *FLP* expression in adult tissues from transgenic mouse lines 4917 (*E*) and 4924 (*F*). Total RNA (20 μg) was fractionated by electrophoresis, transferred to nitrocellulose, and assayed for hybridization to ^{32}P-labeled *FLP* probe 3; lower panels show ethidium bromide staining to document RNA loading. (*E* and *F*) Lanes: 1, testes; 2, brain; 3, heart (degraded sample in *F*, therefore repeated in lane 10); 4, intestine; 5, kidney; 6, liver; 7, lung; 8, spleen; 9, ovary; 10, heart (see lane 3 in *E*); 11, quadriceps and hamstring muscles; 12, gastrocnemius and soleus muscles; 13, uterus. Positions of 28S and 18S rRNAs are indicated. (*G*) Assay for Flp function. Primary EF cultures were prepared from hemizygous h*ACTB::FLP* transgenic embryos as described (29). Cultures derived from five different transgenic mouse lines (4917, 4924, 4925, 4927, 4921), and one nontransgenic line (−), were transfected with 3 μg of target pNEOβ-GAL (13) followed 48 hr later by histochemical X-Gal stain (30). Maximal activity, as indicated by the number of blue cells, was observed in cultures derived from mouse lines 4917 and 4924. Similar results were obtained following transfection with pFRTZ. (*H*) Expression of *FLP* in EF cultures correlates with activity observed in transfection assay. Total RNA (20 μg) was separated and hybridized to ^{32}P-labeled *FLP* probe 3; ethidium bromide staining of gel in lower panel shows equivalent RNA loading. Two independent cultures from each *FLP* mouse line were analyzed: (lanes 1 and 2) h*ACTB::FLP* mouse line 4917, (lanes 3 and 4) line 4924, (lanes 5 and 6) line 4925, (lanes 7 and 8) line 4927, (lanes 9 and 10) line 4921, and (lane 11) nontransgenic negative control.

combined transgenes were detected in this singly transgenic F_3 mouse indicating that recombination was incomplete; a subset of the 30 *FRTZ* transgenes in tandem array underwent recombination.

Conditional Expression of Flp Can Induce Regulated Rearrangement of Target Sequences in Differentiating EC Cells. Controlling expression of the *FLP* transgene is a way to restrict recombination, and therefore gene activation or deletion, to specific cell populations. I investigated whether Flp recombination could be induced in a differentiating EC cell culture system by using *Wnt-1* regulatory sequences (37) to express *FLP* (see Fig. 1*B* for the Wnt1::FLP transgene). RA can induce pluripotent P19 EC cells to differentiate into a mixed population of fibroblasts, astrocytes, and neural cells (38, 39). *Wnt-1* expression is likely induced specifically in neural derivatives, paralleling that seen in embryos where *Wnt-1* mRNA is detected in differentiating neuroectoderm (40).

P19 cells were transiently transfected with target plasmid, target plus phACTB::FLP, or target plus pWnt1::FLP; 0.5 μM RA or control diluent was added to the monolayer 24 hr later.

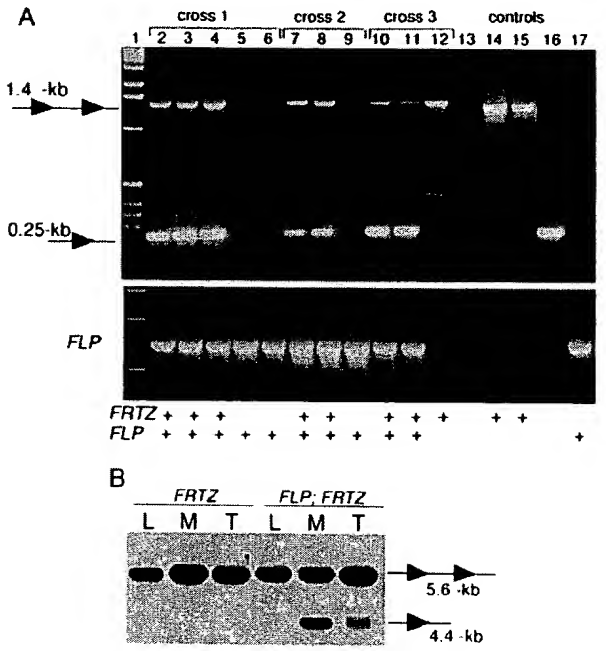


FIG. 4. Dose-dependent Flp recombination in genomic DNA isolated from tissues of doubly transgenic mice. (*A*) Identification of recombined *FRTZ-product* transgenes by PCR amplification of tail DNA. Primers (SD49/SD68) used in this assay flank the FRT-cassette (see Fig. 1*A*); amplification of the target *FRTZ* transgene yields a 1.4-kb fragment and *FRTZ-product*, a 0.25-kb fragment. Parallel reactions using *FLP*-specific primers (SD41/SD42) are shown below. Brackets group littermates from three distinct recombinase target crosses; control single transgenic parental samples and *FRTZ-product* DNA are shown on the right: lanes: 1, 1-kb ladder; 2–6, *FRTZ-5000* × *FLP-4917*; 7–9, *FRTZ-4999* × *FLP-4924*; 10–12, *FRTZ-4999* × *FLP-4917*; 13, no DNA; 14, single transgenic *FRTZ-5000* parental sample; 15, single transgenic *FRTZ-4999* parental sample; 16, *FRTZ-product* DNA; and 17, single transgenic *FLP-4917* parental sample. Genotypes as determined by independent PCR reactions (*FRTZ*, SD23/SD26; *FLP*, SD24/SD25; see Fig. 1) are indicated by plus signs. Sequence analysis of the 0.25-kb product showed precise site-specific recombination (data not shown). (*B*) Correlation between the amount of recombination product and the level of *FLP* RNA expressed in a given tissue. Southern blot analysis using probe 1 (see Fig. 1*A*) of *Bam*HI/*Sca*I-digested genomic tissue DNA (10 μg) isolated from a doubly transgenic (*FLP-4917*; *FRTZ-4999*) mouse or singly transgenic (*FRTZ-4999*) littermate. The expected unrecombined (5.6-kb) and recombined (4.4-kb) fragments within the context of the four-copy array are depicted on the right. Tissue samples include liver (L), muscle (M), and testes (T). For the amount of *FLP* RNA detected in each tissue see Fig. 3*E*; lane 6, liver; lane 12, muscle; lane 1, testes.

Following 5 days of RA treatment, β-Gal activity was assessed by histochemical X-Gal staining. Neural induction was monitored by morphology (the presence of long cellular processes) and culture senescence, as well as by induction of endogenous *Wnt-1* mRNA.

β-Gal activity was detected in target plus pWnt1::FLP cotransfections only following RA induced differentiation (Fig. 5 *C* and *F*). Similarly, endogenous *Wnt-1* expression was absolutely dependent on RA. Low levels of *Wnt-1* transcripts were first detected by Northern blot hybridization after 4 days of RA treatment; no *Wnt-1* RNA was detected in untreated cells (data not shown). As predicted by the nature of the h*ACTB* regulatory sequences, β-Gal-positive cells were observed in the target plus phACTB::FLP cotransfections independent of RA (Fig. 5 *B* and *E*). The target plasmid alone showed no activity (Fig. 5 *A* and *D*). In addition to demonstrating regulated rearrangement of target sequences, these results define a temporal relationship between *FLP* expression

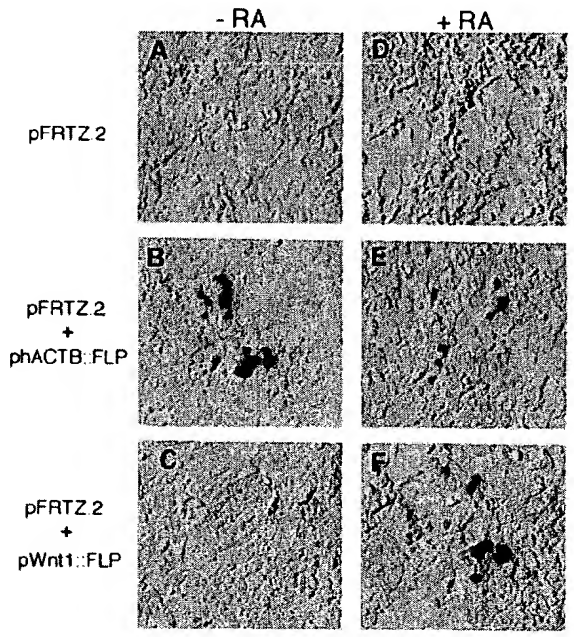


FIG. 5. Regulated Flp recombination in differentiating embryonal carcinoma cells. P19 EC cell monolayers were transiently transfected with the indicated plasmids and induced to differentiate by treatment with RA (38, 39). Target plasmid pFRTZ.2 is identical to pFRTZ except it contains the HSV-tk gene inserted between FRTs. Histochemical X-Gal staining was performed 5 days later. (*A–C*) Exposure to control diluent; (*D–F*) exposure to 0.5 μM RA.

and completed recombination. β-Gal activity, reflecting Flp recombination, was observed in the target plus pWnt1::FLP cotransfection within 24 hr of first detecting *Wnt-1* transcripts, and by inference *Wnt1::FLP* mRNA.

DISCUSSION

This study demonstrates that Flp can effectively recombine target DNA in ES cells, EC cells, and transgenic mice. I have shown that Flp can direct site-specific and heritable DNA recombination in the mouse, and regulated (inducible) recombination in differentiating EC cells. These properties indicate that Flp can be used to make directed modifications of the mouse genome.

Using this Flp system, recombination of an extrachromosomal target can occur in ES cells with an efficiency similar to that previously observed in mouse embryonal carcinoma (F9) cells (13) and in monkey (CV-1) and human (293) embryonic kidney cells (13, 16). Because the efficacy of Flp recombination estimated here (30–78%) is comparable to that reported for Cre (40–80%; ref. 41), it is likely that this Flp system can be exploited to similarly manipulate ES cell chromosomal DNA. Toward this end, Fiering *et al.* (42) has recently employed a more elaborate two-step selection scheme where Flp-mediated deletion of an integrated selectable marker gene (*PGK-neo*) was reported to occur in 90% of Flp-expressing ES cells.

In the mouse, I have shown that Flp expression is necessary and sufficient for excisional recombination of FRT target sequences. Because recombination was detected at all three chromosomal sites assayed, it is likely that most chromosomal transgenes will be accessible to Flp function. The extent of recombination observed in a given tissue correlated directly with the overall amount of *FLP* mRNA detected in that tissue; it is important to note that this type of tissue analysis presents an average and therefore may underestimate the maximal recombination achieved in a specific cell type. Nonetheless, these results define a dose-effect relationship that suggests that

different degrees of recombination can be attained by varying the strength and specificity of the sequences used to express *FLP*. For some experiments, complete (quantitative) recombination may be needed. The results presented here suggest that one means to achieve this is to increase the level of *FLP* expression. Alternative strategies include identifying Flp variants with higher activity in mammalian cells, or to enhance the nuclear localization of Flp.

The finding that Flp can be generally expressed in the mouse without adverse effects suggests that Flp recombination between random sequences in the mouse genome is rare. If high levels of illegitimate (non-FRT) recombination were occurring due to Flp expression, abnormalities would be expected in *FLP* founders or offspring. No adverse effects were detected. This result suggests that Flp can be used to mediate recombination in a variety of cell types.

Flp-mediated excisional recombination is sufficiently dose sensitive that recombination can be regulated in differentiating EC cells in culture. This was evident from examination of RA-treated P19 cells in which the *Wnt-1* promoter was used to express *FLP*. The temporal induction of *Wnt-1* transcripts following RA-induced differentiation indicates that recombination occurred relatively quickly: *FLP* expression, recombination of the target transgene to reconstitute a functional *lacZ* gene, and subsequent β-Gal production occurred within 24 hr. These results demonstrate that regulated rearrangement of a target sequence can be achieved.

The demonstration that Flp can excise DNA in mice and that the recombination product is heritable, suggests that Flp will be useful to study cell lineages. Considering this potential application, the initial test recombination substrate was designed to indicate and "remember" a recombination event by the irreversible gain of β-Gal activity (dependent only on constitutive promoter activity). Mice transgenic for this target should have the capability of marking cell lineages following introduction of Flp by crossing. Toward this end, mice transgenic for Wnt1::FLP have been generated; by crossing to an "optimal" target mouse, cells originating from the dorsal aspect of the developing central nervous system are predicted to be marked. Although all three *FRTZ* target lines analyzed here were competent for recombination, none of the recombined target alleles were sufficiently active to allow cell marking by X-Gal stain (unpublished observations). The lack of β-Gal activity associated with the observed recombination most likely reflects a position effect on transgene transcription exerted by the genomic integration site since only one in four control *FRTZ-product* mouse lines expresses β-Gal (unpublished observations). Such sensitivity to chromosomal context is also supported by the variation in transcript profiles observed when using the same h*ACTB* regulatory sequences to direct *FLP* expression (two of five lines showed general expression in this study). It is likely that by screening more *FRTZ* target loci, a chromosomal integration site will be identified that can support similarly general *lacZ* expression following Flp recombination.

Together, these findings demonstrate that Flp can serve as a tool to alter the mouse genome. By employing both Flp and Cre, it should be possible to engineer multiple independent recombination reactions (gene activation or deletion events) in mice.

I thank E. Robertson for ES cells, M. Zhang for pSLhβAPr-lacZ-pA, A. McMahon for pWEXP2, S. Hachenberg for technical assistance, A. Pinder for oligonucleotide synthesis, A. Fire, G. Seydoux, C. Thompson, and the reviewers for helpful comments. Transgenic mice were generated in conjunction with the National Institute of Child Health and Human Development transgenic mouse development facility, contract N01-HD-0-2911, in support of DNX, Inc. This work was supported by National Institutes of Health Grant R01-HD-30830, the John Merck Fund, and the Carnegie Institution of Washington. S.M.D. is a Helen Hay Whitney fellow.

1. Broach, J. R., Guarascio, V. R. & Jayaram, M. (1982) *Cell* **29,** 227–234.
2. Cox, M. M. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 4223–4227.
3. Vetter, D., Andrews, B. J., Roberts-Beatty, L. & Sadowski, P. D. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 7284–7288.
4. Abremski, K. & Hoess, R. (1984) *J. Biol. Chem.* **259,** 1509–1514.
5. Stark, W. M., Boocock, M. R. & Sherratt, D. J. (1992) *Trends Genet.* **8,** 432–439.
6. Kilby, N. J., Snaith, M. R. & Murray, J. A. H. (1993) *Trends Genet.* **9,** 413–421.
7. Golic, K. G. & Lindquist, S. (1989) *Cell* **59,** 499–509.
8. Buenzow, D. E. & Holmgren, R. (1995) *Dev. Biol.* **170,** 338–349.
9. Struhl, G. & Basler, K. (1993) *Cell* **72,** 527–540.
10. Golic, K. G. (1991) *Science* **252,** 958–961.
11. Chou, T. & Perrimon, N. (1992) *Genetics* **131,** 643–653.
12. Xu, T. & Rubin, G. M. (1993) *Development (Cambridge, U.K.)* **117,** 1223–1237.
13. O'Gorman, S., Fox, D. T. & Wahl, G. M. (1991) *Science* **251,** 1351–1355.
14. Jung, S., Rajewsky, K. & Radbruch, A. (1993) *Science* **259,** 984–987.
15. Ludwig, D. L. & Stringer, J. R. (1994) *Somatic Cell Mol. Genet.* **20,** 11–25.
16. Logie, C. & Stewart, F. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 5940–5944.
17. Lakso, M., Sauer, B., Mosinger, B., Lee, E. J., Manning, R. W., Yu, S.-H., Mulder, K. L. & Westphal, H. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 6232–6236.
18. Orban, P. C., Chui, D. & Marth, J. D. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 6861–6865.
19. Gu, H., Marth, J. D., Orban, P. C., Mossmann, H. & Rajewsky, K. (1994) *Science* **265,** 103–106.
20. Kühn, R., Schwenk, F., Aguet, M. & Rajewsky, K. (1995) *Science* **269,** 1427–1429.
21. Ramirez-Solis, R., Liu, P. & Bradley, A. (1995) *Nature (London)* **378,** 720–724.
22. Zhang, M., Kim, H., Marshall, H., Gendron-Maguire, M., Lucas, D. A., Baron, A., Gudas, L. J., Gridley, T., Krumlauf, R. & Grippo, J. F. (1994) *Development (Cambridge, U.K.)* **120,** 2431–2442.
23. Gunning, P., Leavitt, J., Muscat, G., Ng, S. & Kedes, L. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 4831–4835.
24. Dymecki, S. M. (1996) *Gene* **171,** 197–201.
25. Thomas, K. R. & Capecchi, M. R. (1987) *Cell* **51,** 503–512.
26. Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A. & McMahon, A. P. (1993) *Cell* **75,** 1417–1430.
27. Hogan, B., Constantini, F. & Lacy, E. (1986) *Manipulating the Mouse Embryo: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 152–203.
28. Robertson, E., Bradley, A., Kuehn, M. & Evans, M. (1986) *Nature (London)* **323,** 445–448.
29. Robertson, E. J. (1987) in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, ed. Robertson, E. J. (IRL, Oxford), pp. 71–112.
30. Sanes, J. R., Rubenstein, J. L. R. & Nicolas, J. F. (1986) *EMBO J.* **5,** 3133–3142.
31. Gorman, C. M., Merlino, G. T., Willingham, M. C., Pastan, I. & Howard, B. H. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 6777–6781.
32. Wilkinson, D. G. (1992) in *In Situ Hybridization: A Practical Approach*, ed. Wilkinson, D. G. (IRL, Oxford), pp. 75–83.
33. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
34. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 7.43–7.52.
35. Burrell, H., ed. (1995) *Epicentre Forum* **2,** 4–5.
36. Feinberg, A. & Vogelstein, B. (1993) *Anal. Biochem.* **132,** 6–13.
37. Echelard, Y., Vassileva, G. & McMahon, A. P. (1994) *Development (Cambridge, U.K.)* **120,** 2213–2224.
38. Rudnicki, M. A. & McBurney, M. W. (1987) in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, ed. Robertson, E. J. (IRL, Oxford), pp. 19–49.
39. Schuuring, E., Deemter, L. V., Roelink, H. & Nusse, R. (1989) *Mol. Cell. Biol.* **9,** 1357–1361.
40. Wilkinson, D. G., Bailes, J. A. & McMahon, A. P. (1987) *Cell* **50,** 79–88.
41. Gu, H., Zou, Y. & Rajewsky, K. (1993) *Cell* **73,** 1155–1164.
42. Fiering, S., Epner, E., Robinson, K., Zhuang, Y., Telling, A., Hu, M., Martin, D. I. K., Enver, T., Ley, T. & Groudine, M. (1995) *Genes Dev.* **9,** 2203–2213.